2012

Isolation, Structure Elucidation and Biological Evaluation of Natural Products From Plants and Fungi

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ISOLATION, STRUCTURE ELUCIDATION AND BIOLOGICAL EVALUATION OF NATURAL PRODUCTS FROM PLANTS AND FUNGI

A Dissertation
presented in partial fulfillment of requirements
for the degree of Doctor of Philosophy
in Pharmaceutical Sciences
The University of Mississippi

by

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August 2012

ABSTRACT

Natural products have provided considerable value for drug discovery over the past half-century. Nearly four fifth of drugs were natural products or inspired by natural compounds. Bioactive secondary metabolites isolated from natural source, either fungal or terrestrial, are relatively safer for human health and the environment and can serve as drug or as drug leads for the development of synthetic or semi-synthetic analogs. Natural products have also been successfully used in agriculture as plant growth regulators, signal transductions, fungicides, herbicides and tranquilizers. In our continued efforts to identify bioactive compounds, seventy-seven plant and fungal extracts were evaluated using a direct-bioautography assay coupled with three *Colletotrichum* species to detect antifungal metabolites. Three crude extracts that showed significant antifungal activity were selected for further bioassay-guided isolation. Various chromatographic techniques including preparative thin layer chromatography, vacuum liquid chromatography, flash chromatography, high pressure liquid chromatography and over pressure layer chromatography were utilized to isolate biologically active secondary metabolites from natural products of fungal and plant origin. Among all the techniques, over pressure layer chromatography were first adopt here for preparative isolation of natural products. It turned out to be a rapid and efficient method of exploiting co-migrating bioactive metabolites, and eleven compounds including two new molecules were isolated by using this technique. The structure of the isolated compounds was elucidated through various spectroscopic methods, including high-resolution mass spectrometry, one and two-dimensional nuclear magnetic resonance

spectroscopy. Finally, the isolated compound was submitted for several *in vitro* assays, including antifungal, herbicidal, antibacterial, and cannabinoid and opioid receptor agonist assays. Some compounds showed potent antifungal activity against strawberry pathogens and one compound also showed good herbicidal and antibacterial activity. These compounds could be exploited as drug leads to potential agrochemicals or pharmaceuticals. All the promising drug leads have been submitted for antifungal testing using 24-well strawberry leaf disc assay.

DEDICATION

I dedicate this dissertation to my beloved family who has been always supporting me and encouraging me with their unconditional love.

LIST OF ABBREVIATIONS

AA-MA	Acetate-malonate pathway
AcOEt	Ethyl acetate
AgNP	Silver nanoparticles
CaGoff	*Colletotrichum acutatum*
CB1	Cannabinoid 1
CB2	Cannabinoid 2
CC	Column chromatography
CD	Circular dichroism
CF63	*Colletotrichum fragariae*
CG162	*Colletotrichum gloeosporioides*
1D	One dimensional
2D	Two dimensional
DCM	Dichloromethane
DDI	Distilled de-ionized distilled
DEPT	Distortionless enhancement by polarization transfer
DGAT	Diacylglycerol acyltransferase
DMEM	Dulbecco's modified eagles' medium
EtOH	Ethanol

Et_2O	Diethyl ether
GPCRs	G protein-coupled receptors
HB	Halichondrin B
HIV	Human immunodeficiency virus
HMQC	Heteronuclear multiple quantum coherence
HR-ESI-MS	High resolution electrospray ionization mass spectroscopy
Htopo I	Human DNA topoisomerase I
HTS	High-throughput screening
ILβ	Interleukin β
IL-4	Interleukin-4
IL-5	Interleukin-5
IL-13	Interleukin-13
MeCN	Acetonitrile
MeOH	Methanol
MES	2-N-morpholino ethanesulfonic acid
MOPS	3-(N-morpholino) propanesulfonic acid
NFκB	Nuclear factor kappa B
NOESY	Nuclear overhauser effect spectroscopy
NMR	Nuclear Magnetic Resonance
PCR	PCR polymerase chain reaction

PDA	Potato dextrose agar
PDB	Potato dextrose broth
PKC	Protein kinase C
PPM	Per million units
PTP1B	Protein tyrosine phosphatase 1B
RH	Relative humidity
RPMI	Roswell park memorial institute
rRNA	Ribosomal ribonucleic acid
SAR	Systemic acquired resistance
SAR	Structure activity relationship
%T	Percent of transmittance
THP	Tetrahydropyran
TLC	Thin-layer chromatography
TMS	Trimethylsilane
TNFα	Tumor necrosis factor α
TOCSY	Total correlation spectroscopy

ACKNOWLEDGMENTS

First of all, I would like to express my deepest gratitude to my advisor, Dr. Stephen J. Cutler, for giving me the opportunity to study in his group, giving me countless encouragement, support, patience and caring, and providing me with a compatible and supportive atmosphere for the research over the past few years. Additionally, his magnetic personality will have a significant positive impact on my career and life. I would also like to thank my research advisor, Dr. David E. Wedge. No words can express my gratitude to him for bringing me to the United States and giving me excellent support and valuable advice for both my research and personal life, and also for his willingness to help with any problems I faced over the years.

I am also very grateful to my committee members, Dr. John M. Rimoldi and Dr. Christopher R. McCurdy for spending their valuable time on reviewing and revising my ORP, prospectus and this manuscript.

I want to give special thanks to Dr. Mohamed Radwan, who has provided me with excellent knowledge in chemistry and spectroscopy, for his invaluable advice and insight during my research work. I would also like to thank Dr. Juan Francisco Leon for his considerate personality, friendship, and valuable guidance in part of my research work. I also want to thank my wonderful colleague, Amer Tarawneh, for his friendship and help in the past few years, especially for his help with organic chemistry.

I greatly appreciate all the help, friendship, technique support and love from the members of Wedge Team. I am deeply grateful to Ms. Linda Robertson for teaching me bioassays of all

kinds, always encouraging me, cheering me up and giving me mother-like love and caring in the past few years. I would like to thank Dr. Nurhayat Tabanca for her precious directions in my research, and her kindness and warm encouragement in my daily life. I want to give my sincere thanks to Dr. Luiz Henrique Rosa for his selfless help in fungus identification and generously teaching me the knowledge of microbiology techniques. I would also like to thank Ms. Ramona Pace for her great assistance in data management and Microtiter assays. I want to thank Ms. Carol Wedge for helping me revising the manuscript. All the experiences and memories in this lab will be greatly treasured at the bottom of my heart.

Also, many thanks to all the faculty, staffs and graduate students in the Department of Medicinal Chemistry and all my friends at Ole Miss for the help, friendship and happiness they have brought to me.

Finally, I am deeply indebted to my parents for their unconditional support, caring and love.

Last but not least, I am deeply grateful to my husband, Wei-Bing Ye, for his faithful love and endless encouragement. Thanks to God for bringing him into my life. He is like the sunshine, warming my life.

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CHAPTER 1 BACKGROUND

1.1 NATURAL PRODUCT-BASED DRUGS OR DRUG LEADS

Natural products refer to small organic chemical molecules produced by plants, fungi, bacteria and lower eukaryotes. They also include peptides, proteins, other larger-molecular-weight toxin from marine sources, and hormones from animals (*e.g.* natural estrogens from pregnant mare urine) [1]. In terms of the availability of starting materials, plants and fungi provide a rich source of numerous pharmaceutical and agrochemical agents. There are around 250, 000 types of plants in the world and it is reported that only 10% of them have been investigated. Fungi are also good source of bioactive compounds and about 95% of fungal species have not been studied [2, 3, 4]. Plants and fungi therefore continue to be a great reservoir for identifying novel bioactive secondary metabolites. Historically, the majority of new drugs were natural products or were inspired by natural compounds [5, 6] and over 120 of the most important medicines, including penicillin (**1**) and cyclosporine (**2**), are obtained from fungal extracts [4] (Figure 1.1).

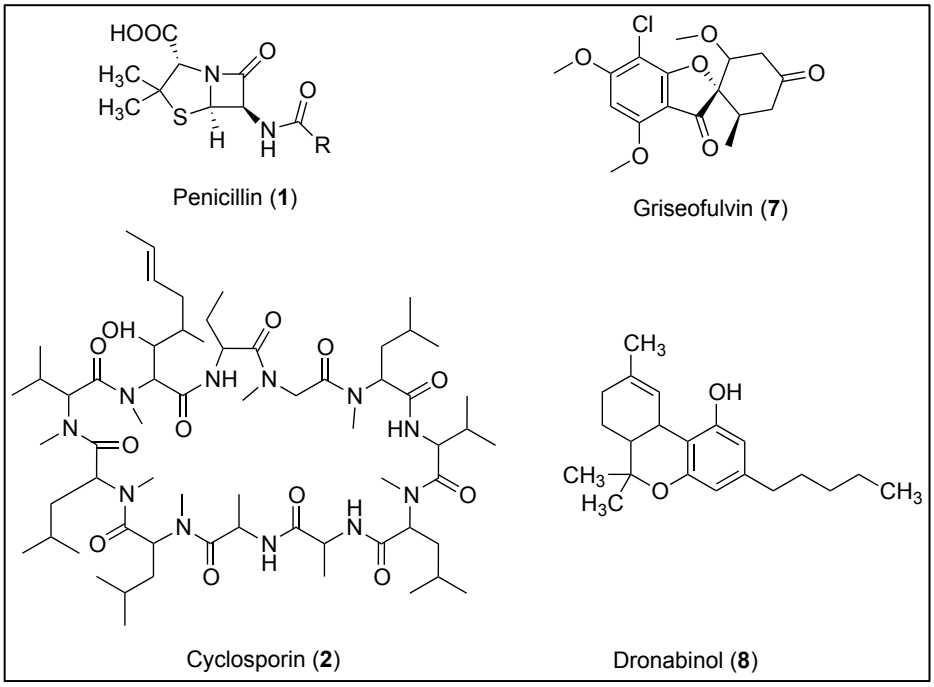


Figure 1.1 Some famous compounds derived from natural source.

Natural products can also serve as lead structures for the development of new synthetic or semi-synthetic analogs. Many of the isolated lead natural compounds are available only in extremely small quantities, especially those from marine organisms such as sponges. The preparation of natural product analogs, which are not naturally occurring, may provide a good yield of desired compounds. And it allows scientists to design and enhance the druglike properties of the medicines that nature has provided, such as bioactivity, solubility and pharmacokinetics. Of importance is that the analogs are easier to synthesize than the parent compounds. Designing unnatural compounds that preserve or even improve biological activity, while removing unnecessary molecular complexity, is the main aim of analog synthesis [7]. Figure 1.2 shows two successful examples of natural product-inspired drug design by structural deletion and unnecessary functionality removal from the parent natural products [8, 9].

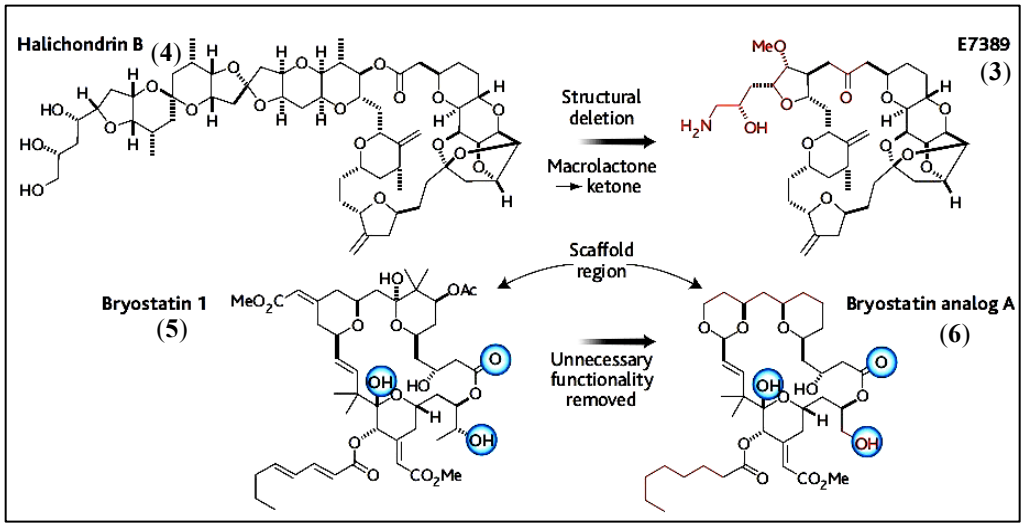


Figure 1.2 Two famous structural modification examples of natural product templates[7].

E7389 (**3**), currently in phase I clinical trials, was generated from the total synthesis of halichondrin B (**4**, HB), a highly cytotoxic marine natural product [8, 10, 11]. Zheng, *et al.* discovered that the deletion of a large region of the molecule did not affect its antimitotic properties [12]. Furthermore, replacing the easily cleaved lactone with a ketone maintained an *in vitro* profile very similar to the parent compound but increased the stability *in vivo*[13]. Another powerful illustration of structure simplification is the development of bryostain analog A. Bryostatin 1 (**5**) is a very promising marine natural product and displays potent biological activity against a variety of human diseases [14]. It is currently in phase II clinical trials as an anticancer agent. Simplification of the scaffold region of Bryostatin 1 resulted in analog A (**6**), which was more potent than the parent compound [15].

Despite these statistics natural products research has experienced a slow decline during the past two decades [15]. The main reason lies in the rapid development of combinational chemistry and high-throughput screening (HTS) in pharmaceutical company [7]. However, untapped biological resources, **"**smart screening**"** methods [16], robotic separation with structural analysis,

metabolic engineering, and synthetic biology have prompted a renewed interest in natural products as a source of chemical diversity and lead compound generation. Advance technologies may provide a vast resource for the future discovery of pharmaceutical and agrochemical agents.

1.2 NATURAL PRODUCT-BASED FUNGICIDES

Natural products have also been successfully used in agriculture as fungicides, herbicides, insecticids, algeacides, plant growth regulators, signal transductions, herbicides and tranquilizers [17]. Griseofulvin (**7**) (Figure 1.1) was the first reported agrochemical fungicide, which is still used today in the treatment of fungal infections [18]. After that, several other plant or bacterial natural products were available on the market as plant protectants through the induction of systemic acquired resistance (SAR) process including Messenger® (EDEN Biosciences, Inc., Bothell, WA) and Serenade® (AgraQuest, Davis, CA), Sonata® (AgraQuest, Davis, CA), and Milsana® (KHH BioSci, Inc., Raleigh, NC). Messenger is derived from a natural protein (harpin), which stimulates a plant's defense and growth mechanism to protect itself from adverse bacterial and fungal diseases. Serenade and Sonata are both microbial-protectants with ability against *Botrytis*, mildews, ruts, blight, and bacterial spot. Milsana is a plant extract widely used for preventive control of powdery mildew. Of special importance, these naturally occurring plant protectants are relatively safer and do not adversely affect the environment and invertebrates due to target specific and biodegradability.

Why do natural products possess such particular specificity and potency compared to artificially synthesized molecules? The answer lies in over millions of years' evolutionary selection by nature. Nature has its own high-through screening process for the optimization of

biologically active compounds. Natural products are endowed with well-defined three-dimensional structures carrying a number of functional groups (hydrogen bond acceptor/donors, *etc.*) that synthetic molecules may lack [19]. Additionally, the structures of the biological receptors of such natural products (*e.g.* protein binding sites) normally have high conservations among proteins of markedly different genetic sequences, such as secondary metabolites that have evolved for a certain purpose by a producing organism may provide different but equally potent effects in other organisms [15].

The common property in both agrochemical and pharmaceutical pursuits is chemistry. Whether the bioactivity of a natural product is for an agrochemical or pharmaceutical application depends on dose. Scientists found that there may be indistinguishable between therapeutic and toxic properties of a compound except by dose. Because of numbers of natural products that have been identified, it was unavoidable that agrochemical and pharmaceutical areas would eventually meld.

1.3 OPIOID AND CANNABINOID RECEPTORS IN DRUG DISCOVERY

Opioid receptors belong to a large family of G protein-coupled receptors. Opioid-binding sites were first discovered in 1973 [20]. Since then, extensive pharmacological studies have been done on a variety of opioid receptor types. To date, three opioid receptors have been identified including μ, κ, and δ. Their activation can be induced by both endogenous opioid peptides and exogenous opioid drugs, such as morphine[21], which is capable of causing analgesia, respiratory depression, euphoria, feeding, the release of hormones, inhibition of gastrointestinal transit, and decreased bowel motility[22, 23]. Currently, the most clinically available opioid analgesics are μ-

agonists that refer to morphine and its analogs. However, their use was restricted because of the side effects including tolerance and physical dependency.

Cannabinoid receptors are also members of a large superfamily of G protein-coupled receptors (GPCRs). There are two subunits of cannabinoid receptors including CB1 and CB2. Previous studies showed that activation of cannabinoid receptors resulted in the stimulation of appetite [24]. Particularly, CB1 agonists were reported to regulate energy input and output through shared central and peripheral mechanisms and CB1 antagonists was expected to contribute long-period efficacy for weight loss [22].

Over the past thirty years, scientists have recognized that development of opioid and cannabinoid receptors targeting drugs is efficient to cure or alleviate CNS symptoms. Although many works have been done in the discovery and development of opioid and cannabinoid-based therapeutics, there are few drugs acting on these two types of receptors. Dronabinol (**8**) is the only drug acting on cannabinoid receptors in the clinic. However, this drug causes many side effects such as confusion, headache, nausea, vomiting and among others [22]. It is apparent that new molecules are needed to continue the fight. Natural products provide a major source of new chemical scaffolds which act on opioid or cannabinoid receptors because small molecules with novel structures and potency can be the biological probes to uncover what is behind these two receptors and the mystery of many related CNS diseases, and also can be the leads for new therapeutics.

1.4 CURRENT SITUATIONS OF COMMERCIAL FUNGICIDES

Strawberry anthracnose is a serious disease in many areas of the world and particularly severe in the southeastern United States where diseases are often enhanced by warm temperature and frequent rains during the harvest season. Anthracnose is a disease that appears as black, sunken lesions on leaves, stems, or fruits and is caused by fungi capable to produce their asexual spores [25]. Strawberry anthracnose disease is mainly caused by filamentous fungi in the genus *Colletotrichum* that often cause typical symptoms of anthracnose diseases on a variety of plants including cereals, grasses, cucurbits, tomatoes, and are serious problems for strawberry (Fragaria × ananassa Duch.) fruits and plant production worldwide [26]. *Colletotrichum* species are defined as destructive pathogens that cause significant economic damage to crops worldwide. The three most common *Colletotrichum* species including *Colletotrichum fragariae* A. N. Brooks, *Colletotrichum acutatum* J. H. Simmonds, and *Colletotrichum gloeosporoides* (Penz.) Penz.&Sacc. can occur singly or in combination [27, 28].*C. acutatum* has a very wide host range and can infect almond, apple, blueberry, and strawberry [29, 30, 31]. Strawberry anthracnose can be devastating since other plant parts may be infected in addition to the fruit. This causes millions of dollars in crop loss each year [32].

Phomopsis viticola Sacc. is another common plant pathogen that causes severe diseases of grapes all over the world, known as Phomopsis cane and leaf spot disease. *P. viticola* (Ellis and Everh.) *B. sutton* can infect canes, leaves, rachis and berries. It was reported that cane infections acted as the primary source of inoculum, while rachis and berry infections caused the highest rate reductions and economic loss [33]. Another important species of *Phomopsis*, *P. obscurans*, causes a disease known as leaf blight of the cultivated strawberry that became a serious problem in 1985.

P. obscurans can infect foliage, runners, petioles, and fruits with a dark brown center surrounded by light-brown rings with purplish halos [34, 35].

Botrytis cinerea Pers.: Fr. is a ubiquitous fungus that infects a wide range of important plants and vegetables, including ornamentals, bulbs, and fruit crops in temperate regions. The pathogen produced profuse conidia and mycelia on the surface of dead and infected stems and calyxes, and cause Botrytis bunch rot of grapes and strawberries [36, 37, 38]. *Fusarium oxysporum* Schlechtend: Fr causes a disease known as Fusarium wilt, which is a major problem in most cotton growing regions of the world. In 1995, it causes huge economic loss in the cotton belt of the United States [39].

Increasing incidence of fungal strain resistance and potential environmental and mammalian toxicities, caused by application of conventional fungicides, are two factors that drive a need to search for new plant protectants [40]. Particularly desirable is exploiting active natural products with new chemical classes that might function by different modes of action than existing fungicides, thus avoiding problems of cross-resistance to current chemicals. Bioactive natural products isolated from plants or terrestrial fungi are safer for human health and the environment and can be used in modern crop protection or serve as lead structures for the development of synthetic or semi-synthetic analogs.

1.5 PRIMARY GOAL OF THIS STUDY

The primary goal of this study was to identify the secondary metabolites responsible for the antifungal activity against plant pathogens from terrestrial and fungal organisms. Some fractions or pure compounds also were tested for their opioid and/or cannabinoid receptor

binding affinity, antibacterial and herbicidal activity. Plant and fungal crude extracts were selected based on the antifungal bioautographic results. 59 plant and fungal extracts were screened using a direct-bioautography assay to detect antifungal metabolites against three *Colletotrichum* species and three of them, *Diospyros virginiana*, *Cladosporium cladosporioides*, and Milogrante (Highlighted in yellow) showed promising antifungal activity and were selected for further isolation study (Table 1.1).

Table 1.1 59 Extracts Screened For Antifungal Activity Using Direct-Bioautography.

Material	Number	Part	Code	Weight (mg)
Anadenanthera spp.	2762:6-10-2005	Seeds (yopo)	A-1	5.9
Berchemia scandens	BUR240394-8	Stem	B-1	5.2
	BUR280394-2	Root	B-2	5.2
	IBEET7618	Shoot	B-3	5.4
	CON021100-4-A	Leaf	B-4	5.9
Croton capitatus	MOBOT145	PX	C-1	5.4
	CON240700-3-A	Stem &Root	C-2	5.4
Diospyros virginiana	CON310700-2-A	Root	D-1	5.4
	CON310700-2-B	Bark	D-2	5.4
	CON310700-2-C	Xylem	D-3	4.9
	CON310700-2-D	Leaf	D-4	5.6
	CON310700-2-E	Fruit	D-5	5.7
	MOBOT165	LF	D-6	5.7
	MOBOT166	ST	D-7	5.5
	MOBOT26	ST	D-8	4.9
	MOBOT328	Fruit	D-9	6.6
	MOBOT329	LF-ST	D-10	5.5
Eupenicillium parvum	MH/But		E-1	4.8
	MH/HAH/EtOAc		E-2	5.2
Hypericum hypericoides	MOBOT1014	PL	H-1	5.3
H. hypericoides	--	PL	H-2	5.4
Hypericum punctatum	--	PL	H-3	5.3
Hypericum stans	MOBOT1016	PL	H-4	4.6
HGC-1		Fungus	H-5	6.1
Ipomoea purpurea	CON200900-3-A	Whole Plant	I-1	6.3
Ipomoea violaceae	2744:4-16-2005		I-2	5.1
Lactuca/Launae taraxacifolia	2761-6-7-05	Stem	L-1	5.2

Lagochilus inebrians	2739:4-12-2005		L-2	4.7
Leonotis leonurus	2727		L-3	5.0
Leonurus cardiaca	2766		L-4	4.4
Nelumbo nucifera	2451	Flower	N-1	5.3
N. nucifera	2453		N-2	4.8
N-2574US		Fungus	N-3	5.2
Solanum carolinense	CON200900-2-A	Whole Plant	S-1	4.9
S. carolinense	3808:7-15-2008	Whole Plant	S-2	5.9
Virola surianmensis	2752:4-19-2005		V-1	5.2
Virola theiodora	2738:4-12-2005		V-2	5.2
NZ-658		Fungus		
NZ-617		Fungus		
ATCC-5907		Fungus		
NZ-647		Fungus		
NZ-656		Fungus		
ATCC-38849		Fungus		
NZ-654		Fungus		
UK-168		Fungus		
NRRL-5507		Fungus		
NZ-574		Fungus		
NZ-648		Fungus		
C. echinulata		Fungus		
ATCC-48726		Fungus		
NRRL-13489		Fungus		
UK-115		Fungus		
Milogrante		Fungus		
NZ-652		Fungus		
NZ-610		Fungus		
ATCC-34655		Fungus		
ATCC-60782		Fungus		
NRLA-17159		Fungus		
ATCC-42761		Fungus		

Crude extracts, fractions, sub-fractions and pure compounds of *Cladosporium cladosporioides* and Milogrante were also tested for opioid and cannabinoid receptor binding affinity. Radio-ligand binding assays for opioid (subtype δ, κ and μ) and cannabinoid receptors (CB1 and CB2) have been a useful tool in the discovery of novel agents. Active compounds were isolated by bioassay-guided fractionation methods. The structures of purified compounds were

elucidated using various NMR spectroscopy and mass spectrometry techniques. Absolute configuration of chiral centers in compounds was assigned and confirmed by X-ray crystallography, and/or chemical derivation. Pure compounds were further tested by 96-microtiter diluted broth assay and 24-well plate strawberry leaf disc assay to determine their *in vitro* potency against plant pathogens.

CHAPTER 2 ANTIFUNGAL METABOLITES FROM THE ROOTS OF DIOSPYROS VIRGINIANA BY OVERPRESSURE LAYER CHROMATOGRAPHY

2.1 INTRODUCTION

2.1.1 Pharmacology and Chemistry of *Diospyros* Species (American Persimmon)

Discovery of new crop protectants from natural products has received considerable interest as alternatives to synthetic agrochemicals for use as pest and disease-control agents. This interest stems from the fact that these are generally safer for human health and the environment. Biologically active natural products can be used in modern crop protection or can serve as lead structures for the development of new semi-synthetic analogs. Our current research efforts are directed to the identification of natural product-based fungicides. Secondary metabolites may be ideal candidates as plant protectants, since their mode of action is selected by nature's evolution that is likely to be different from existing agents, thus avoiding the problem of resistance of the fungi to current fungicides.

Diospyros belongs to the family Ebenaceae which consists of three genera and ca. 500 species of trees and shrubs [41], of which more than 350 species are distributed in tropical and subtropical regions worldwide [42, 43]. *Diospyros* is the largest and the most economically important genus of Ebenaceae. Most of them have a good yield of edible fruits, ebony and

valuable timbers. *D. kaki* is the most well known species, which was originated in East China and has been cultivated in Japan for centuries [44]. The mixture of the leaf extract of *D. kaki* and jasmine has been the major constituent of anti-tobacco smoking candies in Japan. Many other *Diospyros* species have been reported to have important biological and pharmacological activities. For example, *D. peregrina* and *D. melanoxylon*, have been used in folk medicine for the treatment of inflammation and urinary discharges, and for enrichment of blood since olden times [45]. Plumbagin, the major component of *D. kaki* [46] is the first bioactive compounds isolated from *Diospyros* species [47]. It was reported to show mosquito antifeedant and insecticidal activities [48]. Various extracts of *Diospyros* species have also been used as medicines in ancient Thailand, Japan, India and China [45]. Famous examples include the uses of alcohol extracts of *D. usambarensis* [49] and *D. zombensis* [50] as fungicidal and molluscicidal agents, and the uses the leaves of *D. diepenhorstii* as piscicidal and molluscicidal agents [45]. The fruits of *D. lotus* have been used as a sedative, antitussive, antiseptic, antidiabetic, antitumor, astringent, laxative, nutritive and as a febrifuge [51].

In the past century, more than 130 *Diospyros* species have been phytochemically screened and interestingly almost all parts (leaves, bark, roots, fruits) of the *Diospyros* species have been detected for chemical constituents and biological evaluation. The most widely distributed component is triterpenes, which was found in above 90% of *Diospyros* plants [45]. All the triterpenes have a pentacyclic core in common and belong to lupane, ursane and oleananes skeletons. The major compounds of lupane class, which have been reported from more than 25 plants, include lupeol, betulin and betulinic acid, with betulinic acid being the first lupane derivative isolated from one *Diospyros* species[52].

Lupeol (lup-20 (29)-en-3β-ol) is a triterpenoid purified from many popular medical plants such as *Diplotropis ferruginea* Benth [53], *Syzygium formosanum* Hay [54], *Pimenta racemosa* var. *ozua* [55], *Tamarindus indica*, American ginseng, and *Crataeva nurvala*, as well as a variety of fruits and vegetables including olive, fig, mango, strawberry, white cabbage, green pepper [56]. Previous studies found that lupeol is a multi-target agent with a broad range of biological activities against inflammation, cancer, arthritis, protozoal, diabetes, heart diseases, renal toxicity and hepatic activity [57]. The major significance of lupeol is that it causes no toxicity to normal cells when administered in even higher doses[58], which illustrates the fact that secondary metabolites extensively present in edible fruits and vegetables, are potentially more safe than synthetic molecules for human use.

The inhibitory effects on inflammation of lupeol have been widely studied under *in vitro* and animal models. Nguemfo *et al.* stated that lupeol showed higher maximum inhibition in an animal model than a well-known phytochemical α-Mangosteen at similar dose (57.14% vs. 38.70%) [59]. Geetha *et al.* reported the use of lupeol in a mice model of arthritis to suppress the phagocytic activity of macrophages, and T-lymphoeytes and CD4+ cell medicated cytokine generation [60]. Furthermore, a recent study conducted by Vasconcelos and coworkers (2008) showed that application of lupeol caused a significant reduction in eosinophils infiltration and in Th2-associated cytokines (IL-4, IL-5, IL-13) levels responsible for the immune response in asthma [53]. Ding and coworkers concluded that lupeol reduced the LPS-induced IL-6 secretion at concentration of 1 μM [60]. The mechanism of anti-inflammatory activity of lupeol has been studied by several groups. Lupeol has the ability to decrease the generation of pro-inflammatory cytokines such as tumor necrosis factor α (TNFα) and Interleukin β (ILβ) [61]. Further studies

showed that lupeol treatment can decrease the levels of type II cytokines IL-4, IL-5, and IL-13 in a bronchial asthma mouse model [59].

Lupeol and its derivatives have also been studied for their great potential as anticancer agents in several cancer cell lines including prostate, skin, pancreatic and breast cancer [56, 62, 63, 64, 65]. It is noteworthy that lupeol has been shown to exhibit strong antineoplastic and anti-proliferative activity under *in vitro* and *in vivo* situations by directly inhibiting tumor growth, cell cycle progression and inducing the apoptosis of tumor cells [66, 67, 68]. Lupeol was also reported to have anti-mutagenic activity and can inhibit chemically induced DNA damage [69, 70, 71]. Recent studies by Prasad and Nigam *et al.* showed that topical treatment of lupeol could prevent 7,12-dimethylbenz [α] anthracene [DMBA]-induced DNA strand break in murine skin[70] and Benzo[α]pyrene[B(α)P]-induced genotoxicity in a mouse model [72]. Lupeol also has great potential to modulate signaling pathways such as nuclear factor kappa B (NFκB) and the phosphatidylinositol 3-kinase [PI3K]/Akt [73]. Taken together, these compelling evidences warrant that lupeol is a really attractive molecule that can be a lead scaffold for synthetic chemistry and we need to pay great attention to it in further drug investigation.

Betulinic acid (3β-hydroxy-lup-20(29)-en-28-oic acid) is a common compound widely distributed in many plant species with a number of pharmacological activities including anti-inflammatory, anticancer, antimalarial, antibacterial, anthelmintic, antioxidant properties and inhibition of human immunodeficiency virus (HIV)[74]. The sources of betulinic acid include *Betula alba* (Betulaceae), *Ziziphus*spp. (Rhamnaceae), *Syzygium*spp. (Myrtaceae), and *Paeonia*spp. (Paeoniaceae)[74, 75]. Hundreds of publications have described the application of

betulinic acid for treatment of cancer [76, 77, 78], viral infections [79], hair loss [80], and other conditions. However, the most regarded activities of betulinic acid are anti-HIV-1 activity and specific cytotoxicity against a variety of tumor cell lines [77].

In 1994, Fujioka *et al.* reported that betulinic acid was capable of inhibiting HIV-1 replication with an IC_{50} value of 1.4 μM [81]. In light of its promising anti-HIV activity, a variety of betulinic acid analogs were subsequently synthesized to enhance the anti-HIV activity. RPR103611 was concluded to be the only low-molecular-weight analog to date that effectively inhibits HIV infection at an envelope-dependent post-binding step with an IC_{50} value ranging between 40 and 100 nM in various *in vitro* cell-based assays [82]. SAR studies showed that the A-ring of betulinic acid was not an ideal site for structure modifications [83]. The mechanism of anti-HIV of betulinic acid and betulin analogs appeared to be associated with viral entry and maturation [75].

A report in 1995 showed that betulinic acid selectively inhibited human melanoma cells grown *in vitro* [84]. The mechanism of anti-tumor activity of betulinic acid was thought to be through induction of apoptosis independent of the cell's p53 regulation [85]. Betulinic acid and its derivatives were found to have a high protein tyrosine phosphatase 1B (PTP1B) and diacylglycerol acyltransferase (DGAT) inhibitory activity [86, 87]. Betulinic acid was reported to show activity against TPA-induced tumors, ovarian, melanoma xenographs in mice models [88]. Because of its selective cytotoxicity against tumor cells and lacking of side effects to normal cells, betulinic acid became a very promising novel chemotherapeutic agent for the treatment of HIV infection and cancer.

Ursane is another class of pentacyclic triterpene that are widespread in the *Diospyros* species. One representative metabolite in this class is ursolic acid (3-β-hydroxy-urs-12-en-28-oic acid), which was isolated in good quantities from many *Diospyros* plants and other tea trees, spice plants such as paulownia [89]. Ursolic acid has been reported to decrease incidence of gastric ulceration induced by pyloric ligation [90], and also inhibit proliferation of multiple myeloma cells [91].

Besides triterpenoids, the uniqueness of *Diospyros* genus is the presence of a large number of napththoquinone metabolites, which all have a juglone framework. 7-methyljuglone (5-hydroxy-7-methyl-1, 4-naphthoquinone) is a chief component in this class, and was previously isolated and identified as an active component from the root extracts of *Euclea natalensis* with titubercular and termiticidal activity [92]. One recent report showed that 7-methyljuglone exhibited intracellular and extracellular inhibition of *M. tuberculosis* comparable to streptomycin and ethambutol [93]. Because of its fairly simple chemical structure and pharmacological effects, many scientists have paid attention to the total synthesis of this compound. It was first synthesized by Cooke *et al.* in 1952 and improved by Musgrave *et al.* in 2001 [94].

Shinanolone (6-methyl-4, 8-dihydroxy-3, 4-dihydronaphthalene) is also a 1,4-naphthoquinone metabolite, which is common in *Diospyros* species. One report showed that shinanolone exhibited anti-mycobacterial activity against *M. tuberculosis* at a concentration of 0.1 mg/mL and antibacterial activity against a variety of test organisms[95,96].

As mentioned before, the most common *Diospyros* naphthoquinone is 7-methyljuglone. Isodiospyrin (5-hydroxy-6-(1-hydroxy-6-methyl-5,8-dioxo-naphthalen-2-yl)-2-methyl-naphthalene-1,4-dione) is a dimer of 7-methyljuglone linked between C-6 and C-8'. It was the first compound isolated from the wood of *Diospyros virginiana* by Fallas and Thomson in 1968 [97] and was reported to show significant cytotoxicity against the HCT-8 colon tumor, P-388 lymphocytic leukemia[K], HEPA-3B hepatoma, KB nasopharynx carcinoma, CLOL-205 colon carcinoma and HeLa cervical carcinoma[L]. Isodiospyrin acts as a novel human DNA topoisomerase I (htopo I) inhibitor through the ability to antagonize camptothecin-induced, htopo I-mediated DNA cleavage and inhibits htopo I by direct binding to htopo I without access to the DNA substrate [98]. Furthermore, isodiospyrin exhibits strong inhibitory effect on the kinase activity of htopo I toward splicing factor 2/alternate splicing factor in the absence of DNA. Thus, these findings have important implications on naphthoquinone and its derivatives' cellular mode of actions, *i.e.* these novel DNA topoisomerase I inhibitors can prevent both DNA relaxation and kinase activities of htopo I.

Diospyrin is a plant-derived bisnaphthoquinonoid compound with less active properties than its racemic isomer, isodiospyrin. It is a dimer of 7-methyljuglone linked between C-2 and C-3'. Diospyrin has been used as a potential lead molecule to new drug against cancer as well as several other diseases like leishmaniasis, trypanosomiasis, malaria and tuberculosis[99, 100, 101, 102]. *Diospyrin* derivatives were found to be cytotoxic with the ability to generate reactive oxygen and to induce apoptosis in human cancer cell lines [103].

Diospyros virginiana L. (named by Carl Linnaeus), also called American persimmon, is

native to North America, and the fruits were reported to show cholesterol-lowering activity, and usefulness to treat bloody stools, thrush, and sore throats [104]. The tree is common in the South Atlantic and Gulf States, especially in the Mississippi River Vally. Traditional uses of the plant include treatment of diarrhea and hemorrhoids, but today it is mainly used for culinary purposes. Excessive consumption of the fruits can cause gastrointestinal disorder [105]. Many *Diospyros* species are a rich source of triterpenes, naphthoquinones, and other naphthalene derivatives [92]. A literature research contains reports of eight compounds, including isodiospyrin, shinanolone [92], diospyrin, 7-methyljuglone [97] and scopoletin [106] isolated from the wood of *D. virginiana*, and lupeol, betulin and betulinic acid isolated from the leaves of *D. virginiana* [107]. However, little attention has been given to chemical fractions from the roots of *D. virginiana*.

The aim of this research was to identify naturally occurring active compounds with antifungal activity against seven common plant pathogens. These include isolates of *Colletotrichum fragariae* A. N. Brooks, *Colletotrichum acutatum* J. H. Simmonds, and *Colletotrichum gloeosporoides* (Penz.) Penz. & Sacc., *Botrytis cinerea* Pers.: Fr, *Fusarium oxysporum* Schlechtend: Fr, *Phomopsis obscurans* (Ellis and Everh.) *B. sutton*, and *P. viticola* Sacc., which cause severe diseases to many crops each year, especially strawberry plants. In this research program, we evaluated 37 plant extracts using a direct- bioautography bioassay to detect antifungal activity against three *Colletotrichum* species. Of these, *D. virginiana* root extracts showed the most promising activity. Based on preliminary screening results, a bioassay-guided fractionation of *D. virginiana* root extract was conducted to isolate and identify the pure metabolites possessing antifungal activity. A preparative overpressure layer chromatography (OPLC) method was successfully used for the separation of two new natural compounds, 4-

hydroxy-5, 6-dimethoxynaphthalene-2-carbaldehyde (**9**) and 12, 13-didehydro-20, 29-dihydrobetulin (**10**) together with nine known compounds, including 7-methyljuglone (**11**), diospyrin (**12**), isodiospyrin (**13**), shinanolone (**14**), lupeol (**15**), betulin (**16**), betulinic acid (**17**), betulinaldehyde (**18**), and ursolic acid (**19**) from the acetone extract of the roots of *D. virginiana*. Their identification was accomplished by 1D- and 2D-NMR (One Dimensional-and two Dimensional- Nuclear Magnetic Resonance) spectroscopy and HR-ESI-MS (High resolution electrospray ionization mass spectroscopy) methods. All the isolated compounds were evaluated for their antifungal activities against *Colletotrichum fragariae*, *C. gloeosporioides*, *C. acutatum*, *Botrytis cinerea*, *Fusarium oxysporum*, *Phomopsis obscurans*, and *P. viticola* using an *in vitro* micro-dilution broth assay. The results indicated that compounds **3** and **5** showed high antifungal activity against *P. obscurans* at 30 μM with 97.0 and 81.4% growth inhibition, and moderate activity against *P. viticola* (54.3 and 36.6%). It appears that an optimized OPLC system offers a rapid and efficient method of exploiting bioactive natural products.

2.1.2. Over Pressure Layer Chromatography (OPLC)

We should keep in mind that a single plant can contain up to several thousand secondary metabolites, so high-performance and rapid separation methods are absolutely needed to be emphasized [95]. Several conventional methods including silica gel, polyamide and reversed-phase liquid chromatography are often used to isolate pure compounds from natural plants, but they are tedious, time-consuming, and require multiple chromatographic steps [108]. Overpressure layer chromatography (OPLC) with a programmable pump equipped to deliver the mobile phase overcomes the problems stated above. OPLC was developed by Tyihák and Mincsovics in the late seventies [109]. A TLC plate is covered by a sheet of flexible material in OPLC and subjected to a high external pressure (Figure 2.1). The high external pressure generated by the

programmable pump pushes the mobile phase through the analytical or preparative adsorbent layer. This allows a faster separation and more compact spots than conventional TLC. Generally, the separation distance of the compounds migrated over the adsorbent is two to five times greater than in conventional TLC. Another advantage of OPLC is that it is a sealed system with less solvent loss as a result of evaporation [110, 111]. Therefore, the resolution is better than the obtained by conventional TLC, especially in the lower and closer R_f range. Compared to conventional methods, OPLC offers a more rapid and reliable method for the isolation of naturally occurring compounds.

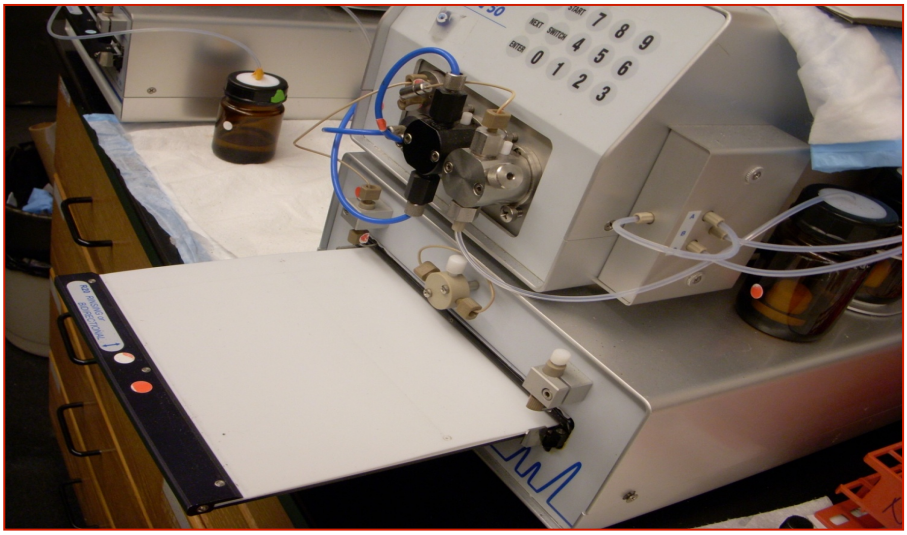


Figure 2.1 OPLC systerm (BIONISIS SA., Le Plessis-Robinson, France).

OPLC was widely used in analytical and preparative applications reported in previous literature[112, 113, 114, 115, 116]. In the present paper, a preparative off-line OPLC technique was used to isolate co-migrating compounds from the roots of *D. virginiana* using transfusion operation (the chamber outlet is open during development). The difference between off-line and on-line OPLC separations lies in the existence of a flow-cell detector. On-line OPLC usually couples to

other chromatographic, electrophoretic, and spectroscopic techniques (*e.g.* OPLC-FTIR, OPLC-UV, OPLC-MS, and OPLC-MALDI-MS). In off-line OPLC separation, there is no flow-cell detector connected to the outlet and the eluent is detected by TLC analysis.

2.2 EXPERIMENTAL SECTION

2.2.1 Apparatus

OPLC: Personal OPLC 50 instrument (OPLC-NIT, Budapest, Hungary), at 50 bars external pressure; OPLC silica gel (SiO_2) layer, 60 F_{254} 20×20 cm on glass plate (LG 011, OPLC-NIT Ltd., Budapest, Hungary) with 200 μM sorbent thickness, 11 μM particle size, 6 nM pore size, and 20×20 cm on aluminum sheet (BSLA 001, OPLC-NIT Ltd., H-Budapest) with 200μM sorbent thickness, 5 μM particle size, 6 nM pore size; an AS-30 sample applicator (DESAGA, Wiesloch, Germany) was used for the sample application. TLC: Classical TLC analysis was performed on silica gel 60 F_{254} 20×20 cm on aluminum sheet (Gibbstown, New Jersey, USA). Detection was carried out under UV light (254 and 366 nM), and visualization with vanillin-H_2SO_4 (1 g of vanillin in 100 mL of 20% H_2SO_4 in EtOH) reagent, followed by heating at 105 °C for 5 min. UV Spectra: Varian Cary 50 spectrophotometer; in MeOH; λ_{max} (log ε) in nm. IR Spectra: Bruker Tensor 27 spectrophotometer; ν in cm^{-1}. ^{1}H- and ^{13}C-NMR spectra: Bruker model AMX-500 and 400 NMR spectrometers with standard pulse sequences, operating at 500 and 400 MHz for ^{1}H, and 125 and 100 MHz for ^{13}C; δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. High resolution electrospray ionization mass spectroscopy (HR-ESI-MS): Micromass Q-Tof Micro mass spectrometer with a lock spray source; in *m/z*.

2.2.2 Chemicals and Solvents

Silica gel column chromatography was performed with silica gel 60 (70-230 mesh, Merk). Pre-coated aluminum silica gel 60 F_{234} 20×20 cm plates (Merck) were used for classical TLC analysis. ACS-grade solvents, acetonitrile (MeCN), acetone, dichloromethane (DCM), chloroform ($CHCl_3$), diethyl ether (Et_2O), ethyl acetate (AcOEt), ethanol (EtOH), *n*-hexane, isopropanol (iPrOH), methanol (MeOH), benzeneand toluene from Fisher Scientific (New Jersey, USA) were used for SiO_2 column chromatography (CC) and TLC separations. HPLC-Grade solvents, acetonitrile (MeCN), chloroform ($CHCl_3$), dichloromethane (DCM), and $H_2O$from Sigma Aldrich (St. Louis, USA) were used for HPLC and OPLC chromatograms.

2.2.3 Plant Material

The roots of *D. virginiana* were collected from the Missouri Botanical Garden, USA, in June 2009. The plant material was identified by Dr. Vaishali C. Joshi, and a voucher specimen CON310700-2-A was deposited with the National Center for Natural Products Research, School of Pharmacy, The University of Mississippi.

2.2.4 Extraction of the Plant Material

Dried powdered roots (66 g) were extracted in a Soxhlet extractor with acetone (600 mL) for 8 h, and the extract was subsequently evaporated under vacuum to yield a dry residue (2 g). The crude extract was fractionated on a SiO_2 column (100 g, 50×5 cm, ChemGlass) using hexane (500 mL), 5% AcOEt in hexane (600 mL), $CHCl_3$ (600 mL), 5% iPrOH in $CHCl_3$ (600 mL), and MeOH (600 mL), resp. Four fractions were obtained in total, Fractions 1-4. Bioautography-guided assay showed that the activity was found to reside in fraction 1 (120 mg) and fraction 2 (240 mg), with some activity in fraction 3 (710 mg), while fraction 4 (800 mg) did not show any

activity against three notorious plant pathogenic fungi of the *Colletotrichum* species. The active fractions were subjected to bioassay directed isolation of the pure isolates (Figure 2.2).

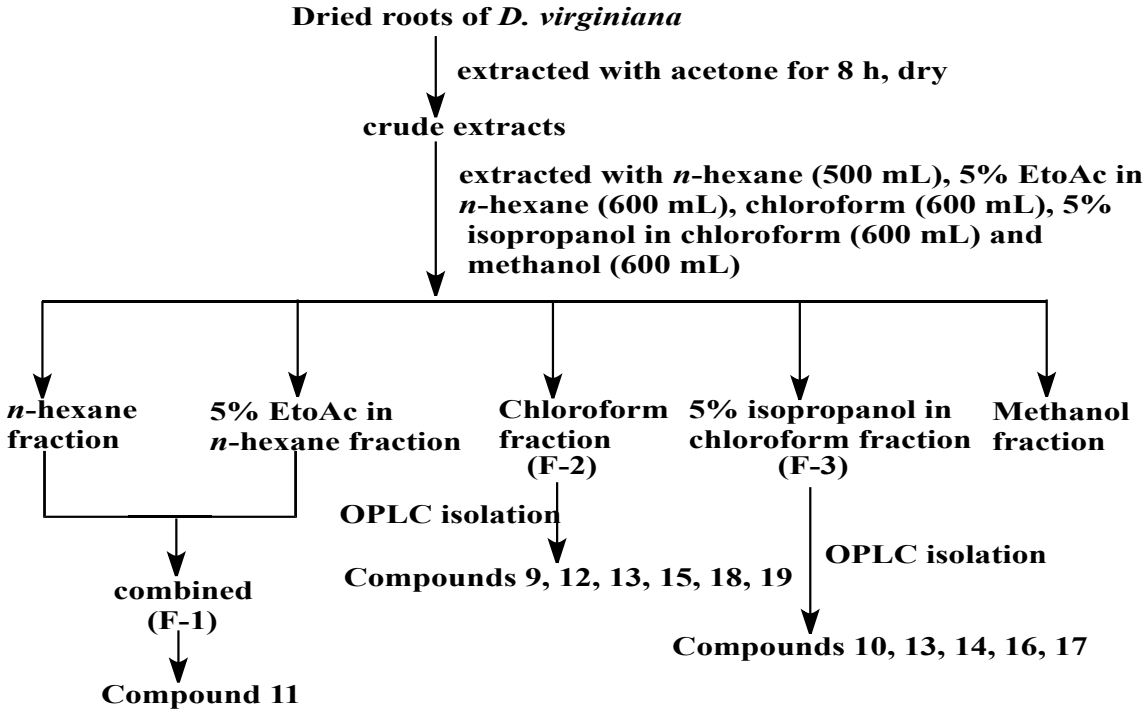


Figure 2.1 Bioassay-guided fractionations of the acetone extract of *Diospyros virginiana* roots.

2.2.5 Isolation of Compound 11 (7-methyljuglone)

Fraction 1(120 g), which is the combination of *n*-hexane and 5% EtOAc in *n*-hexane fractions contains two compounds with different R_f values. This fraction was applied to preparative TLC plates and developed with chloroform-acetone (9:1, v/v) to yield one major compound **11**, 7-methyljuglone [114].

2.2.6 Selection of OPLC Conditions for Fractions 2 and 3

Fraction 2 (240 mg) and Fraction 3 (800 mg) were applied and purified by off-line

OPLC-transfusion technique. In order to get good separation, many mobile phases (*n*-hexane-ethyl ether, toluene-EtOAc, *n*-hexane-EtOH,*n*-hexane-EtOAc, DCM, DCM-MeOH, $CHCl_3$-acetone, $CHCl_3$-EtOAc, *n*-hexane-EtOAc, DCM-EtOAc, EtOAc-*n*-hexane, DCM-ethyl ether, $CHCl_3$-MeOH, $CHCl_3$-acetonitrile, $CHCl_3$-isopropyl, toluene-EtOAc-*n*-hexane-formic acid) with different ratios were tested and compared. Chromatographic results showed that two mobile phases, DCM (100%) and n-hexane-ether (6/4, v/v) were preferred by Fraction 2 separation. Four mobile phases, $CHCl_3$-EtOAc (95/5, 98/2, v/v), n-hexane-ether (6/4, v/v), $CHCl_3$-acetonitrile (98/2, v/v) and toluene-EtOAc-*n*-hexane-formic acid (60:10:10:1, v/v/v/v) were suitable for Fraction 3 purification.

2.2.7. Isolation of Compounds 9, 12, 13, 15, 18, 19 From Fraction 2 Using OPLC

A portion of Fraction 2 (20 mg) was purified by OPLC with DCM as the eluent. Elution conditions were as follows: flash volume, 300 μL; eluent volume, 30,000 μL; flow-rate, 500 μL min^{-1}; development time, 3606 s; external pressure, 50 bars. 88 Subfractions (1 mL/fraction) were obtained in total, of which sub-fractions 49–54 were detected as the same compound and combined together to give betulinaldehyde (**18**) (0.8 mg), amorphous yellow solid. The structure was identified by NMR spectroscopy and by comparison with reported data [117]. Sub-fractions 72–88 afforded ursolic acid (**19**; 1.3 mg), amorphous white solid. The structure was identified by the comparison with previous data[118]. Sub-fractions 28–37 were combined to give a mixture of three compounds (7.9 mg) and further purification was performed with *n*-hexane-ether (6/4, v/v, 20,000 μL; flash volume, 300 μL; flow-rate, 500 μL min^{-1}; development time, 2406 s) as eluent and 66 Sub-fractions were obtained, among which Sub-fractions 10**'**–18**'** furnished lupeol (**7**; 2mg) by the spectroscopic data and previously study [119]. Sub-fractions 19**'**–66**'** (marked as Fraction A) were combined to give a mixture of 3.6 mg containing four compounds with close R_f

values in several TLC systems (SiO_2; hexane/EtOAct 3:2, CH_2Cl_2/iPrOH 9:1, $CHCl_3$/MeOH 19:1). To obtain enough material (Fraction A) for further purification, OPLC procedures were repeated six times under the same conditions, then Fraction A multiplied to 19.6 mg and then was purified by OPLC with DCM (100%, 20,000 µL; flash volume, 300 µL; flow-rate, 400 µL min^{-1} time, 3007 s). Sub-fractions 27”-33” were combined and identified as lupeol. Sub-fractions 12**’’** was detected as pure on the TLC plate and showed yellow fluorescence under UV366. This pure compound was identified as 4-hydroxy-5, 6-dimethoxynaphthalene-2-carbaldehyde (**9**; 1mg), which was reported here for the first time. Sub-fractions 9**’’**–11**’’**were combined and submitted for NMR analysis. It was elucidated as diospyrin (**12**; 2 mg) and it was in agreement with the previous data[115]. Sub-fractions 14”-21” were combined to give amorphous yellow solid that was elucidated as isodiospyrin (**13**; 2 mg) by the NMR analysis and comparison to reported data [114, 116].

2.2.8 Chromatograms of Pure Compounds 9, 12, 13, 15, 18 and 19

Off-line OPLC separation of Fraction 2 led to isolation of compounds **9**, **12**, **13**, **15**, **18** and **19** which correspond to 4-hydroxy-5, 6-dimethoxy-2-naphthaldehyde (**9**), diospyrin (**12**), isodiospyrin (**13**), lupeol (**15**), betulinaldehyde (**18**), and ursolic acid (**19**). Four µL of 20 mg/mL with 6 mm band size of compounds **9**, **12**, **13**, **15**, **18**, **19** and Fraction 2 were applied at 30 mm measured from the bottom edge of the adsorbent layer (200 × 200 × 0.2 mm). The distance between two bands was 10 mm. OPLC infusion development (the outlet is closed) was developed twice with DCM (100%, HPLC-grade) as eluent. The elution was conducted as follows: volume of rapid eluent admission, 300 µL; development volume, 4887 µL; eluent flow-rate, 400 µL min^{-1}; development time, 740 s; external pressure, 39 bar. They were developed twice with DCM (100%, HPLC-grade) using off-line OPLC infusion development and the

chromatogram can be seen in Figure 2.3. Compound **1** showed yellow florescent under UV_{366}. Figure 2.3c showed that the compounds had very similar Rf values (**19,** 0.42; **18**, 0.53; **13,** 0.55; **15**, 0.64; **9**, 0.68; **12**, 0.75) on the chromatogram, however, they were all nicely isolated from each other by the application OPLC.

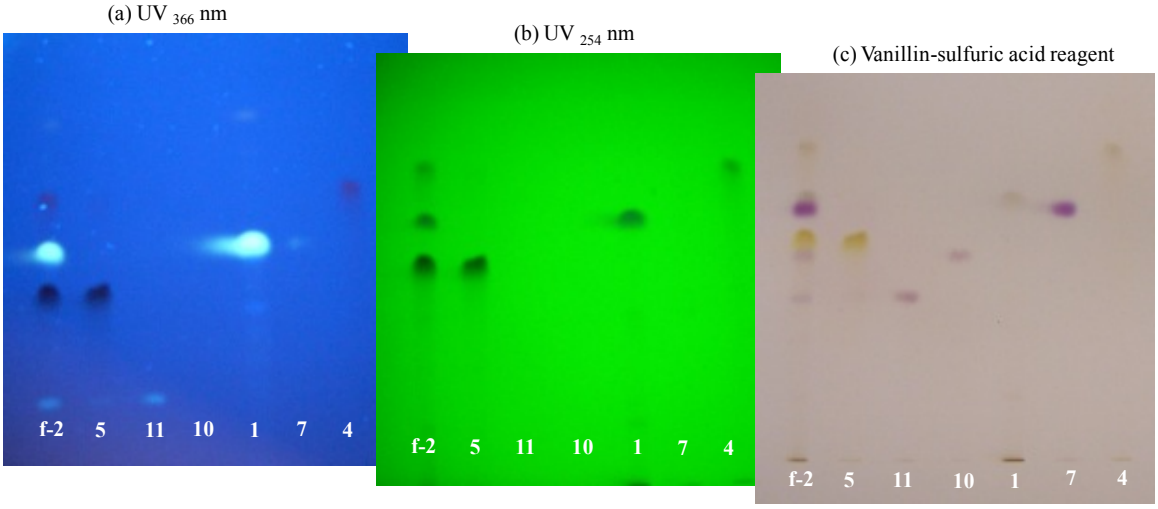


Figure 2.3 Chromatograms of off-line OPLC separation of six pure compounds from faction 2 under UV with developed by DCM (a & b), and visualized by vanillin-sulfuric acid plus heating (c). The bold numbers represent the compounds in Figure 2.8.

2.2.9. Isolation of Compounds 10, 13, 14, 16, 17 From Fraction 3 Using OPLC

Fraction 3 (24 mg) was subjected to OPLC with $CHCl_3$-AcOEt (95: 5, v/v, 40,000 µL) with flash volume, 300 µL, flow rate, 500 µL min^{-1} and elution time 4806 s. One hundred and twenty-three sub-fractions (1 mL/fraction) were collected in total. Sub-fractions 54–66 yielded botulin [120] (**16**; 3.4 mg). Sub-fractions 10-22 were combined to give 2.3 mg of mixture of two compounds and labeled as fraction B, which was further purified by *n*-hexane-ether (6/4, v/v) using OPLC to yield isodiospyrin that was previously isolated in Fraction 2. Sub-fractions 67–90 were combined to give 7.4 mg of mixture containing two or three compounds and one of them

showed yellowish-green fluorescence under UV_{366} and were marked as fraction C. Fraction C was performed with 20,000 μL of toluene-EtOAc-*n*-hexane-formic acid (60/10/10/1, v/v/v/v) solvent system with flash volume 300 μL, flow rate 250 μL min^{-1} and total elution time 4812 s. Ninety-three sub-fractions were totally obtained and detected by TLC. Sub-fractions 18'-26' were tested as the same compound that was elucidated as amorphous white solid, betulinic acid [121] (**17**). Fraction C (39 mg, multiplied by repeating the above OPLC procedures five times) was purified using OPLC with $CHCl_3$-acetonitrile (98/2, v/v) with flash volume 300 μL, flow rate 300 μL min^{-1} and total elution time 4806 s. Sub-fractions 79'-87' were combined to give a yellowish-green compound, which was identified as shinanolone [122] (**14**, 1.5 mg). The other minor compounds were in such small quantities that they were impossible to isolate. To obtain enough material for further purification, flash chromatography of fraction 3 (400 mg; BIOTAGE (Isolera One); $CHCl_3$/AcOEt (0–5%; 200 mL) and $CHCl_3$ (100%, 201 mL–640 mL); flow rate, 5 mL min^{-1}; Si, SNAP 25 g column) was conducted. A total of 81 sub-fractions (1 mL/fraction) were collected. Sub-fractions 38 –51 were combined to yield a mixture (30 mg). This mixture was subjected to the OPLC ($CHCl_3$/MeCN 49: 1 (40,000 μL); flash volume, 300 mL flow rate, 250 mL/min total elution time, 9624 s). 183 Sub-fractions (1 mL/fraction) were obtained. Subfractions 100–112 were combined and identified as botulin (**16**; 2mg). Sub-fractions 136–144 yielded betulinic acid (**17**; 1mg). Sub-fractions 114–126 gave the new triterpene, 12, 13-didehydro-20, 29-dihydrobetulin (**10**; 1 mg) as a natural product [123].

2.2.10 Chromatograms of Pure Compounds 10, 13, 14, 16 and 17

Compounds **10**, **13**, **14**, **16** and **17** represent 12, 13-didehydro-20, 29-dihydrobetulin (**10**), isodiospyrin (**13**), shinanolone (**14**), betulin (**16**), and betulinic acid (**17**). They were developed with $CHCl_3$-acetonitrile (97:3, v/v) using off-line OPLC infusion development and the

chromatogram is showed in Figure 2.4. Two µL of 20 mg/mL with 4 mm band size of compounds **10**, **13**, **14**, **16**, **17** and fractions 3 were applied and the distance between two bands was 8 mm. Off-line OPLC infusion development was developed with $CHCl_3$-acetonitrile (97/3, v/v, HPLC-grade) as eluent. The elution was conducted as follows: volume of rapid eluent admission, 150 µL; development volume, 2787 µL; eluent flow-rate, 200 µL min^{-1}; development time, 843 s; external pressure, 39 bar. Compound **6** showed yellow-greenish color under UV_{366}. These co-migrating compounds had similar R_f values (**17**, 0.19; **14**, 0.21; **10**, 0.33; **16**, 0.35; **13**, 0.7). Particularly, compounds **10** and **16** had very similar R_f values on the OPLC plate. Forced flow and longer development distances in the OPLC applications clearly revealed **10** and **16** separations with R_f values 0.33 and 0.35, respectively.

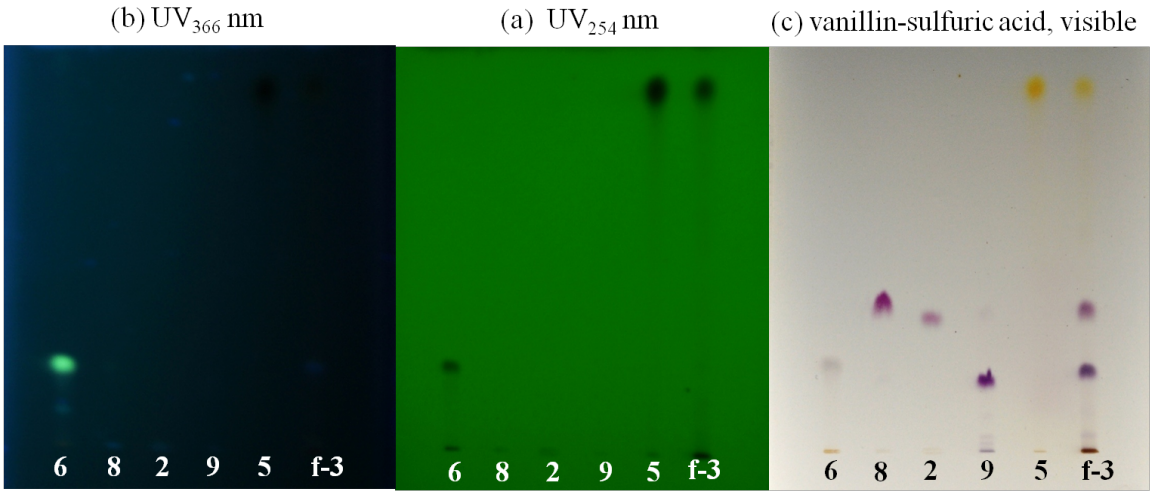


Figure 2.4 Chromatograms of off-line OPLC separation of five pure compounds from fraction 3 under UV with developed by $CHCl_3$-acetonitrile (97:3, v/v) (a & b), and (c) visualized by vanillin-sulfuric acid reagent plus heating.

2.3 BIOLOGICAL EVALUATION

2.3.1 Direct-Bioautography

2.3.1.1 Pathogen production

Three *Colletotrichum* species including *C. fragariae* (isolate CF63), *C. acutatum* (isolate CaGoff), and *C. gloeosporioides* (isolate CG162) obtained from Dr. B. J. Smith (USDA, ARS, Small Fruit Research Station, Poplarville, MS) were used for direct-bioautography study. Isolate CF63 is one of the most infective isolates infecting strawberry plants and causing both crown and fruit rot [124]. CaGoff, CF63 and CG162 were used as standard test isolates because of our comprehensive knowledge of these isolates and their fungicide sensitivity profiles in both bioautography and microtiter formats.

2.3.1.2 Inoculum preparation

Fungal cultures were initiated on ½ strength potato dextrose agar (PDA, Difco, Detroit MI) from spores stored in sterile 10% glycerol RPMI (Roswell Park Memorial Institute, Gibco) buffer with MOPS (3-(N-morpholino) propane sulfonic acid) at -80°C. Fifty µL suspended spore solution was inoculated on PDA plate using crossed inoculation method. Inoculated plates were incubated at 24 ± 2°C under cool-white fluorescent lights (55±5 µmols/m^2/s^1) with a 12 h photoperiod. *Colletotrichum* cultures were subcultured or harvested from PDA every 7-10 days. Conidia were harvested by flooding plates with 3-5 mL of sterile distilled water and dislodging conidia by softly brushing the colonies with an L-shaped glass rod. Conidial suspensions were filtered through sterile Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA) spread on a sterile funnel to remove mycelia. Conidia suspensions were adjusted with sterile distilled de-

ionized distilled (DDI) water to a concentration of 1.0×10^6 CFU (colony forming units)/ mL (19, 20). Conidia concentrations were determined photometrically from a standard curve based on the percent of transmittance (%T) at 625 nm. Conidial suspensions of each *Colletotrichum* species (CF63, CaGoff and CG162) were then adjusted to a concentration of 3.0×10^5 conidia/mL with liquid potato-dextrose broth (PDB, Difco). Using a chromatographic sprayer, each 250 μM silica Gel GF Uniplate (Analtech, Inc. Newark, DE) thin-layer chromatography (TLC) plate was sprayed lightly (to a damp appearance) three times with the conidial suspension [125].

2.3.1.3. Bioautography

Antifungal compounds were indicated by inhibition of fungal growth on chromatographic plates using modifications of TLC bioautographic assays [126, 127, 128]. The acetone extract of *D. virginiana* roots was dissolved in chloroform at 20 mg/mL concentrations. Using a disposable glass micropipette for each sample, 4 μL and 8μL of each test compound were placed on the TLC plate in a grid format to achieve a final amount of 80 and 160 μg/spot.

To detect biological activity directly on the TLC plate, silica gel plates were sprayed with either of the three spore suspensions. Inoculated plates were placed in a 30 cm×13 cm×7.5 cm 398-C moisture chamber (Pioneer Plastics, Inc. Dixon, KY) and incubated in a growth chamber at 24±1°C and 12 h photoperiod under 60±5 μmols/m^2/s^1light. Inhibition of fungal growth was observed 4 days after treatment. Sensitivity of each fungal species to each test compound was determined by comparing the size of inhibitory zones.

Technical fungicide grade standards benomyl, cyprodinil, azoxystrobin, and captan (Chem Service Inc., West Chester, PA) were used as positive controls at 2 mM in 2 µL of 95% ethanol. Benomyl (a benzimidazole inhibitor of microtubule formation) and captan (a phthalimid with multisite inhibition) are the commercial fungicides labeled for control on strawberry anthracnose. Azoxystrobin (a methoxyacrylate preventing the generation of ATP) is commercially used to control *Colletotrichum* and *Botrytis* diseases. Cyprodinil (a methionine biosynthesis inhibitor) is mainly used for control of cereal diseases and of *Botrytis* spp. on grapes and vegetables [129]. For the antifungal assay 20 mg/mL of crude extracts or semi-purified fractions were applied to precoated Si gel TLC plates, developed with $CHCl_3$, and dried for complete removal of solvents. The chromatograms were sprayed with a spore suspension of *C. fragariae*, *C. acutatum*, and *C. gloeosporioides* in potato dextrose broth (PDB) and incubated for 4 days in a moistened chamber at 25 °C, following the previously reported procedure (Figure 2.5). The result showed that mild polar compounds appear to be responsible for antifungal activity.

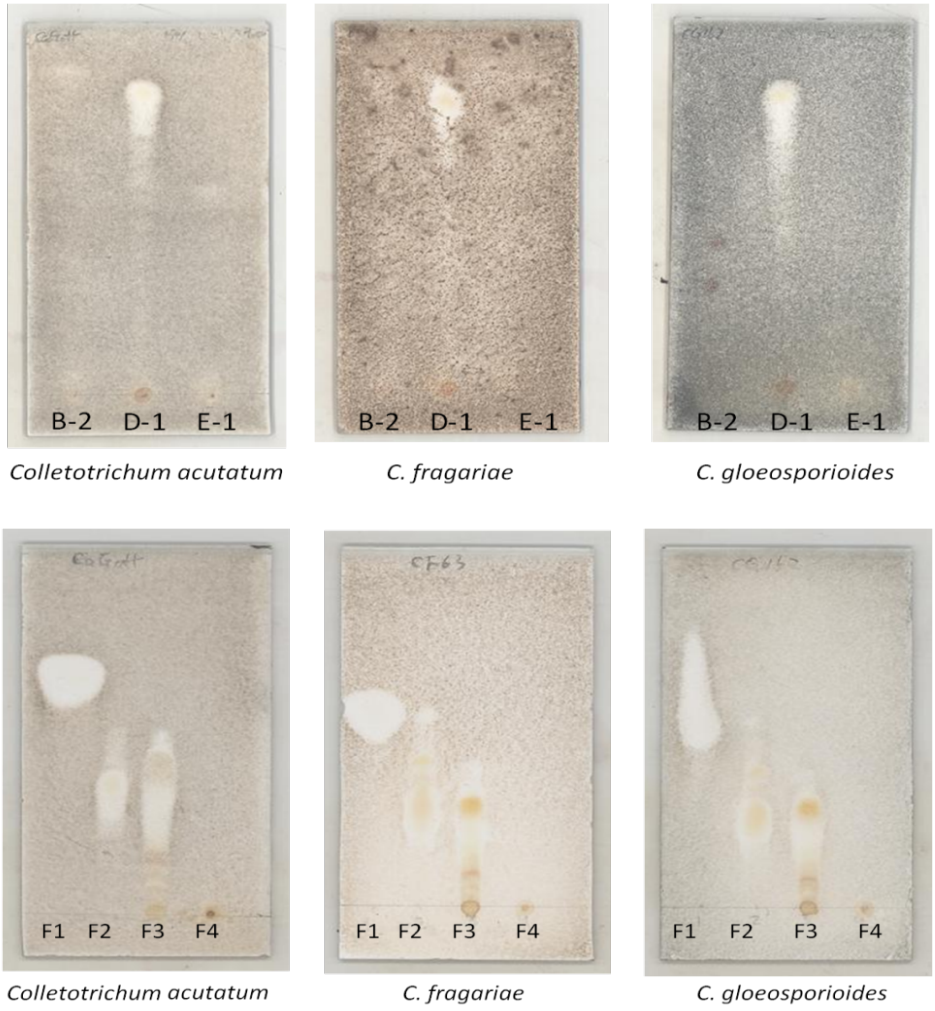


Figure 2.5 Bioautogrphic results of root crude extracts and fractions of *Diospyros virginiana*. *Diospyros virginiana* (D-1) root extracts showed promising antifungal activity against three *Colletotrichum* species. Fraction 1-3 exhibited antifungal activity against plant pathogens, and Fraction 4 did not exhibit any activity.

2.3.2 Microtiter Assay

A standardized 96-well micro-dilution broth assay developed by Wedge and Kuhajek [130] was used to evaluate the antifungal activity of pure compounds from *D. virginiana* that were

identified as active by bioautography (Figure 2.6)

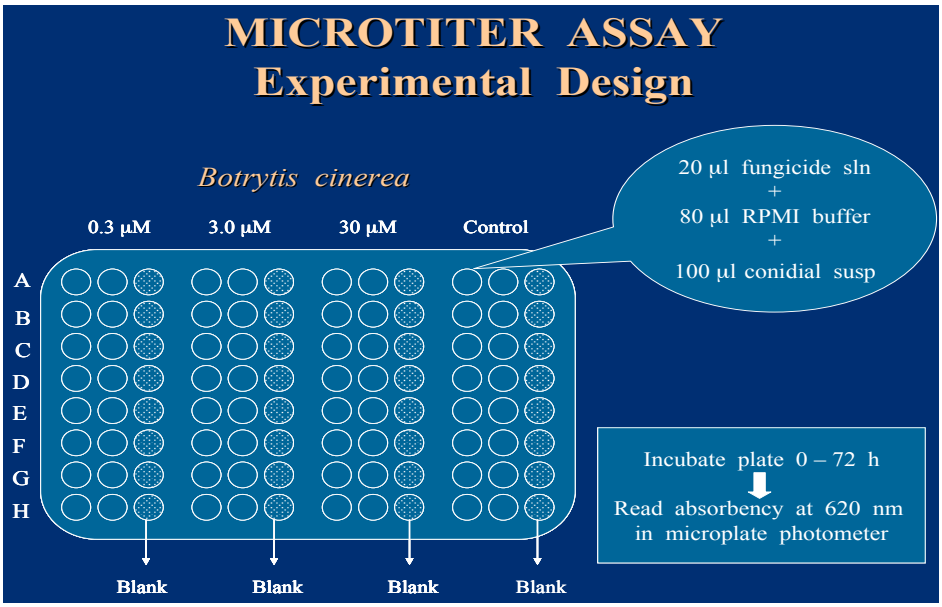


Figure 2.6 Experimental design of a standardized 96-well micro-dilution broth assay.

Isolates of *C.acutatum*, *C. fragariae*, *C. gloeosporioides*, *B. cinerea*, *F.oxysporum*, *P. obscurans*, and *P. viticola* were used to evaluate the antifungal activity of the test compounds using *in vitro* micro-dilution broth assay. Each fungus was challenged in a dose-response format using test compounds where the final treatment concentrations were 0.3, 3.0 and 30.0 µM. Technical grade commercial fungicides captan, azoxystrobin, and benomyl, which represent three different modes of actions, were used as positive fungicide standards. Each compound was evaluated in duplicate and the experiment was performed three times in time. Mean absorbance and standard errors were used to evaluate fungal growth after 48 and 72 h, except for *P. obscurans* and *P. viticola* (120 and 144 h).

2.4 RESULTS AND DISCUSSION

The roots of *D. virginiana* were extracted with acetone in a Soxhlet extractor. The constituents of this extract were mainly separated by OPLC. An OPLC system is considered as the bridge between TLC and HPLC, which uses a programmable pump to push the mobile phase through the "flat column" and the forced flow leads to a faster separation and improved efficiency than capillary flow. Furthermore, the use of longer separation distances increases zone capacity compared to conventional TLC. OPLC offers a sealed system where compounds can be developed with less solvent in less time. OPLC stands out from a number of isolation techniques due to its flexibility to stop the elution process and visualize the separation of compounds under UV during the process of development. According to observation result, we can decide to continue the elution and/or change the elution solvent or convert a double-development method. Rapid speed of elution through OPLC columns limits the diffusion effects of the compounds, making the separation of closely related compounds more purified than the one from classical TLC, thereby concentrating bioactive compounds into a smaller more concentrated zone or band and thereby enhancing direct bioautography detection sensitivity. Another advantage of OPLC is that the eluent flow rate is adjustable through the adjustment of three different solvent systems (A, B, and C) for isocratic or step-wise gradient runs[131, 132, 133]. However, the OPLC column has to be used in right direction. Figure 2.7 showed the conditions of OPLC chromatogram for Fraction 2 and its compounds in two directions (A & B). Apparently, B showed a longer and cleaner separation among compounds, which indicates that the right developing direction of OPLC plate needs to be emphasized. OPLC coupled with direct-bioautography offers a powerful tool to separate co-migrating bioactive compounds for the discovery of natural product for pest management.

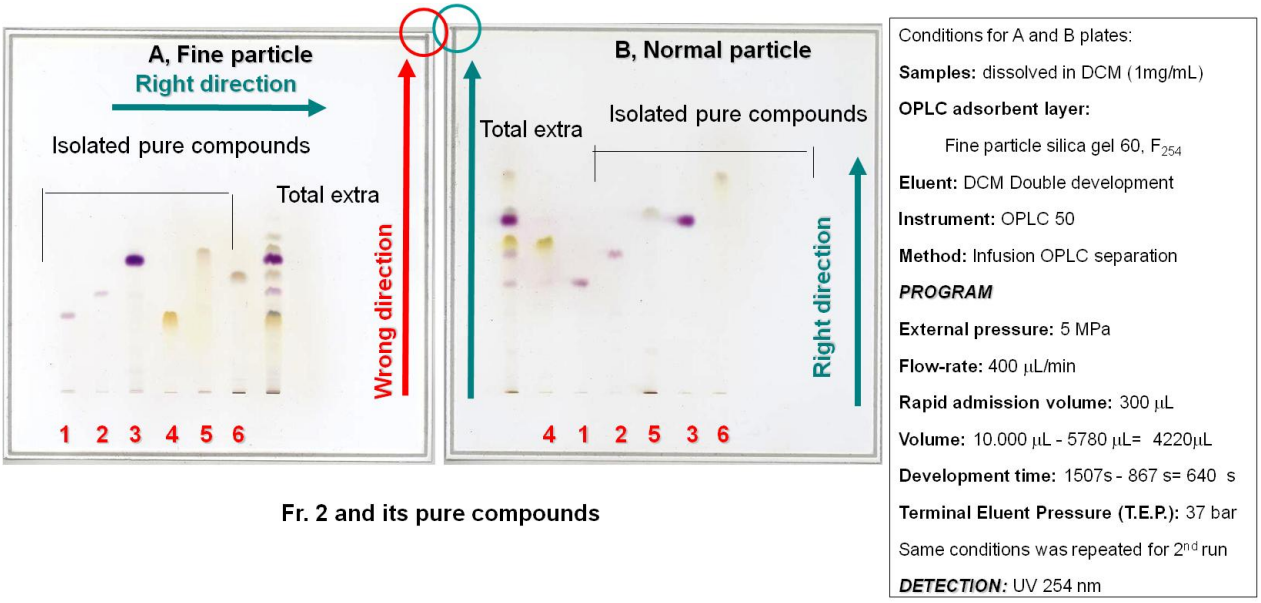


Figure 2.7 Fraction 2 and its pure compounds.

A new naphthalene derivative, 4-hydroxy-5, 6-dimethoxynaphthalene-2-carbaldehyde (**9**), and a triterpene, 12,13-didehydro-20, 29-dihydrobetulin (**10**) were isolated from a natural source for the first time, along with nine known compounds, 7-methyljuglone (**11**), diospyrin (**12**), isodiospyrin (**13**), shinanolone (**14**), lupeol (**15**), betulin (**16**), betulinic acid (**17**), betulinaldehyde (**18**), and ursolic acid (**19**). Although they are common in most of the Ebenaceae family [45], compounds **18** and **19** were isolated for the first time from *D. virginiana*. The structures of the known compounds were confirmed by comparison of their spectroscopic data (^{1}H- and ^{13}C-NMR, and MS) with literature values (Figure 2.8).[134]

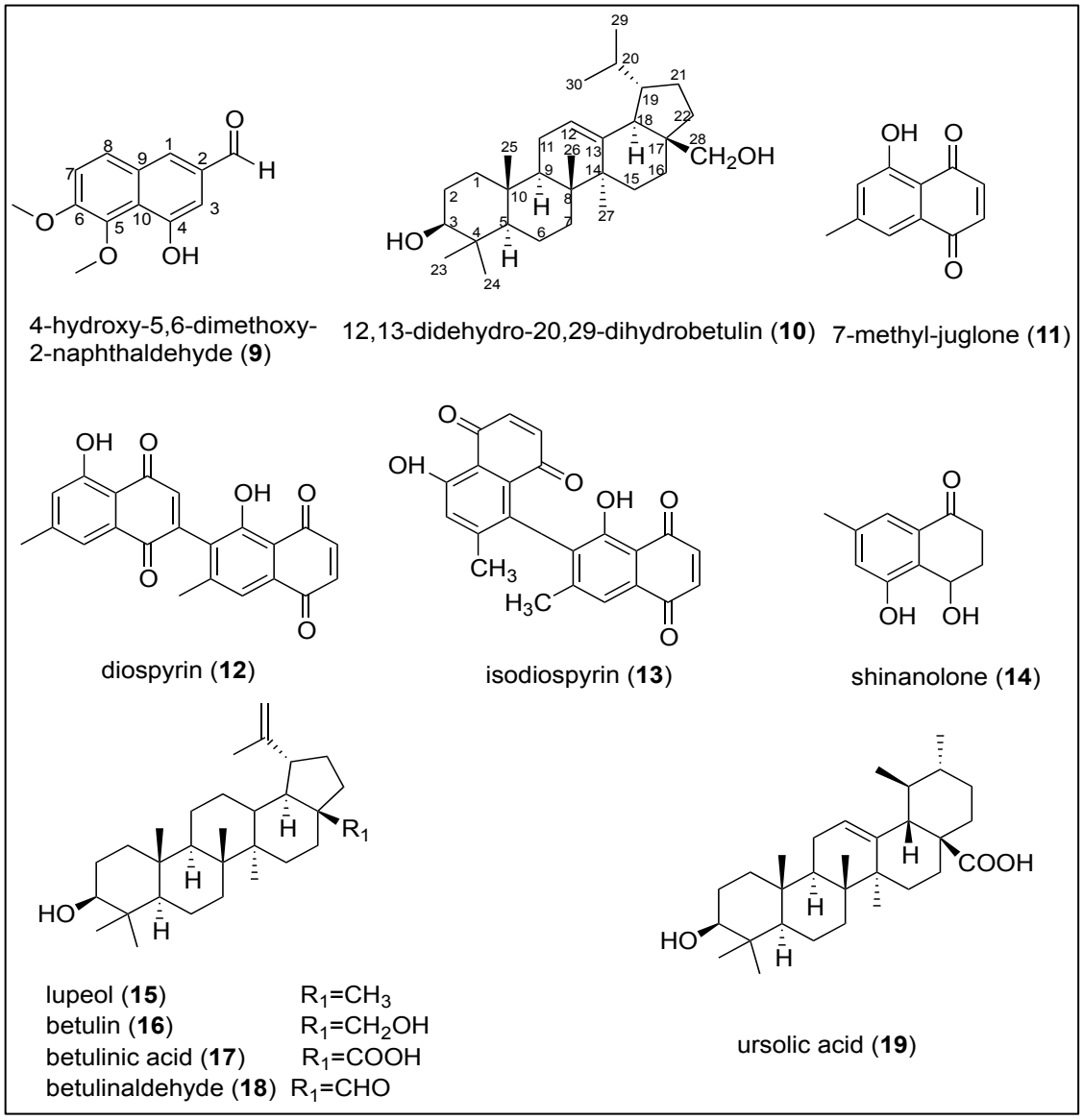


Figure 2.8 Isolated compounds from the roots of *Diospyros virginiana*.

The HR-ESI-MS of compound **1** indicated a molecular formula $C_{13}H_{12}O_4$, and its IR spectrum exhibited characteristic absorption bands for OH and conjugated C=O groups. The ^{1}H-NMR spectrum showed signals for an CHO group at δ (H) 10.03 ppm, and an aromatic *AB* system at δ (H) 7.37 (*d*, *J*=9.0) and 7.78 (*d*, *J*=9.0). These data suggested a naphthalene-2-carbaldehyde structure with two MeO groups (δ (H) 4.06 and 4.13 ppm), and an OH substituent (δ (H) 9.75 ppm). The ^{13}C-NMR spectrum showed 13 signals of which ten were typical aromatic

C-atoms. Assignment of structure was accomplished by HMBC experiment. The NOESY experiment showed a correlation between the MeO group signal at δ (H) 4.06 and H-C (7) signal (δ (H) 7.37 (*d*, *J*=9.0 Hz)), and MeO signal at δ (H) 4.13 and the OH signal at δ (H) 9.75 ppm, corroborating the location of the MeO groups at C (5) and C (6). Thus, **9** was characterized as 4-hydroxy-5, 6-dimethoxynaphthalene-2-carbaldehyde.

Compound **10** was obtained as a white solid. The molecular formula was determined as $C_{30}H_{51}O_2$ by HR-ESI-MS (*m/z*443.3874 ([M+H]+)). The ^{13}C-NMR data of **10** indicated the presence of 30 C-atoms comprising seven CH_3 groups, ten CH_2 groups, seven CH groups, and six quaternary C-atoms. TheCH_3 groups resonated at δ (H) 0.79 (*s*, 6 H), 0.93 (*d*, *J*=6.8, 6 H), 0.98 (*s*, 3 H), 0.99 (*s*, 3 H), and 1.10 (*s*, 3 H) in the ^{1}H-NMR spectrum, indicating the lupane triterpene skeleton for **10** [135]. The ^{13}C-NMR spectrum also revealed the presence of one olefenic CH group (δ(C) 125.0), which correlated in the HMQC spectrum with the H-atom at δ (H) 5.20 (H-C (12)). The latter H-atom showed HMBC correlation with C (9), C (14), and C (18), evidencing the C (12)=C (13) bond. This was confirmed by the COSY correlation between H-C (12) (δ (H) 5.20) and CH_2 (11) (δ (H) 1.90), and further supported by the HMBC correlation of Me (27) (δ (H) 1.10) and C (13) (δ(C) 136.7) (Figure 2.9). From these data, the compound **10** was deduced to be 12,13-didehydro-20, 29-dihydrobetulin. This is the first report of **10** from a natural source together with its full spectral data, although it has been prepared synthetically, and only the ^{1}H-NMR data were reported [123].

4-hydroxy-5, 6-dimethoxynaphthalene-2-carbaldehyde (**9**): Yellow amorphous solid; UV (MeOH): λ_{max} nm (log ε): 375 (4.1), 320 (4.2), 270 (3.8); IR (neat): ν_{max} 3332, 2923, 2851, 2360, 2341, 1687, 1372, 1274, 1055 cm^{-1}; ^{1}H- NMR (500 MHz, $CDCl_3$): δ 4.06 (3H, *s*, OMe), 4.13

(3H, *s*, OMe), 7.29 (1H, *s* br, H-C (3)), 7.37 (1H, *d*, *J*= 9.0 Hz, H-C (7)), 7.78 (1H, *d*, *J*=9.0 Hz, H-C (8)), 7.81 (1H, *s* br, H-C (1)), 9.75 (1H, OH-C (4)), 10.03 (1H, *s*, H-C (11)); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 56.67 (OMe), 62.20 (OMe), 106.14 (C(3)), 115.46 (C(7)), 121.49 (C(10)), 126.02 (C(1)), 127.19 (C(8)), 130.120 (C(9)), 134.09 (C(2)), 143.46 (C(5)), 149.95 (C(6)), 154.28 (C(4)), 191.79 (C(11)); HR-ESI-MS: *m/z* =233.0812 ($[M + H]^+$, $C_{13}H_{13}O_4$; calc. 233.0814).

12,13-didehydro-20, 29-dihydrobetulin (=(3β)-Lup-12-ene-3, 28-diol; **10**): White amorphous solid; $[\alpha]^{20}_D$ = +79.2 (*c* 0.05, MeOH); IR (KBr): ν_{max} 3340, 2940, 2867, 1446, 1372, 1027 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.73 (1H, *d*, *J*=7.2 H-C(5)), 0.79 (6H, *s*, Me-24, Me-26), 0.89 (1H, *m*, , H-C(20)), 0.93 (6H, *d*, *J*= 6.8 Hz, Me-29, Me-30), 0.98 (3H, *s*, *J*=9.0 Hz, Me-25), 0.99 (3H, *s*, Me-23), 1.10 (3H, *s*, Me-27), 1.20-1.81 (8CH_2), 1.90 (2H, *m*, H_2-C(11)), 3.19 (1H, *d*, *J*=10.4, H-C(28^a)), 3.22 (1H, *m*, H-C(3)), 3.53 (1H, *d*, *J*=10.4, H-C(28b)), 5.13 (1H, br. *s*, H-C(12)); ^{13}C NMR (100 MHz, $CDCl_3$): δ 15.6 (C(29)), 15.7 (C(24)), 16.7 (C(25)), 17.3 (C(26)), 18.3 (C(6)), 21.3 (C(30)), 23.2 (C(21)), 23.3 (C(27)), 23.4 (C(11)), 25.9 (C(2)), 27.2 (C(15)), 28.1 (C(23)), 30.6 (C(16)), 32.8 (C(7)), 35.2 (C(1)), 36.9 (C(14)), 38.7 (C(22)), 38.0 (C(4)), 39.3 (C(20)), 39.4 (C(19)), 40.0 (C(10)), 42.0 (C(8)), 42.3 (C(17)), 47.6 (C(18)), 54.0 (C(9)), 55.1 (C(5)), 69.9 (C(28)), 79.0 (C(3)), 125.0 (C(12)), 136.7 (C(13)). HR-ESI-MS *m/z* = 443.3874 ($[M + H]^+$, $C_{30}H_{51}O_2$; calc. 443.3889).

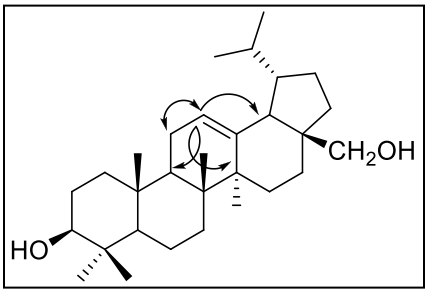
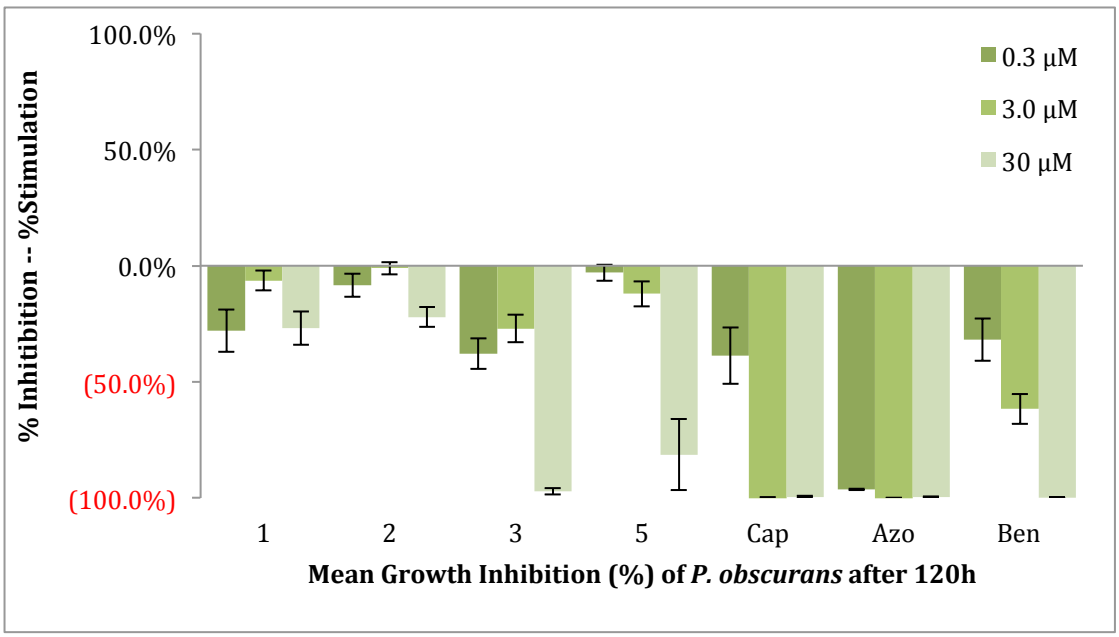


Figure 2.9 Relevant ^{1}H-^{13}C HMBC (→) and ^{1}H-^{1}H COSY (↔) correlations of **10**.

The antifungal activities of compounds **9**-**19** were evaluated using a 96-well micro-dilution broth assay against the plant pathogens *B. cinerea*, *F. oxysporum*, *P. obscurans*, *P. viticola*, and three *Colletotrichum* species. *Phomopsis* species were the most sensitive fungi to these compounds. 7-Methyljuglone (**3**) and isodiospyrin (**5**) showed the highest antifungal activities against *P. obscurans*. Compound **3** showed 97.0% growth inhibition of *P. obscurans* at 30 μM at 120 h, whereas compound **5** showed 81.4% growth inhibition. The antifungal activities of **11** and **13** at 30 μM against *P. viticola* were 53.4 and 57.7%, respectively.

The new compounds 4-hydroxy-5, 6-dimethoxynaphthalene-2-carbaldehyde (**9**) and 12, 13-didehydro-20, 29-dihydrobetulin (**10**) at 30 μM showed weak antifungal activities with 26.9 and 22.1%, respectively (Figure 2.10). Compounds **11** and **13** at 30 μM caused 54.3 and 36.6% growth inhibition of *P. viticola* at 120 h. Any test compound possessing < 50% growth inhibition at 30 μM is considered to have a weak antifungal activity in this bioassay. Compound **9** was more active against *P. viticola* at 120 h than at 144 h. This response is often observed when an inducible enzyme system is turned on, and a compound is detoxified by the fungus. However, the upward slope of the graph for compound **9** in Figure 2.11 from the low to high concentration may be indicative of precipitation in the aqueous micro-dilution broth assay (there is not enough

amount of compound **9** for another microtiter assay). Because the microtiter plate reader measures changes in optical density, it does not discriminate between fungal growth and precipitation. Compound **9** appears to have come out of solution at the higher concentration (30 μM) in the *P. viticola* testing (Figure 2.11). Lipophilic compounds are problematic in *in vitro* aqueous bioassays, and follow-up antifungal testing will take place using a detached leaf bioassay [136]. The graphical results indicated that compounds **10**, **11**, and **13** appear to remain soluble at 120 and 144 h. Commercial fungicides captan and benomyl are significantly more active than any of the compounds tested. Azoxystrobin, which is commercially used to control *Colletotrichum* and *Botrytis* diseases, shows poor activity against *P. viticola*. Both captan and azoxystrobin show 100% growth inhibition at 3 μM against *P. obscurans*. Although once an excellent agent for controlling anthracnose and other diseases of strawberries and ornamentals, resistance developed by pathogens has resulted in benomyl being less useful [137]. While the test compounds appear inherently antifungal, we hypothesize that these compounds are probably present in the plant as constitutive defense compounds that act to deter infection or fungal growth. Since these compounds were found without elicitation, they are probably constitutive in nature [138,139] and may have a potential role in preventing fungal infection in *D. virginiana.*



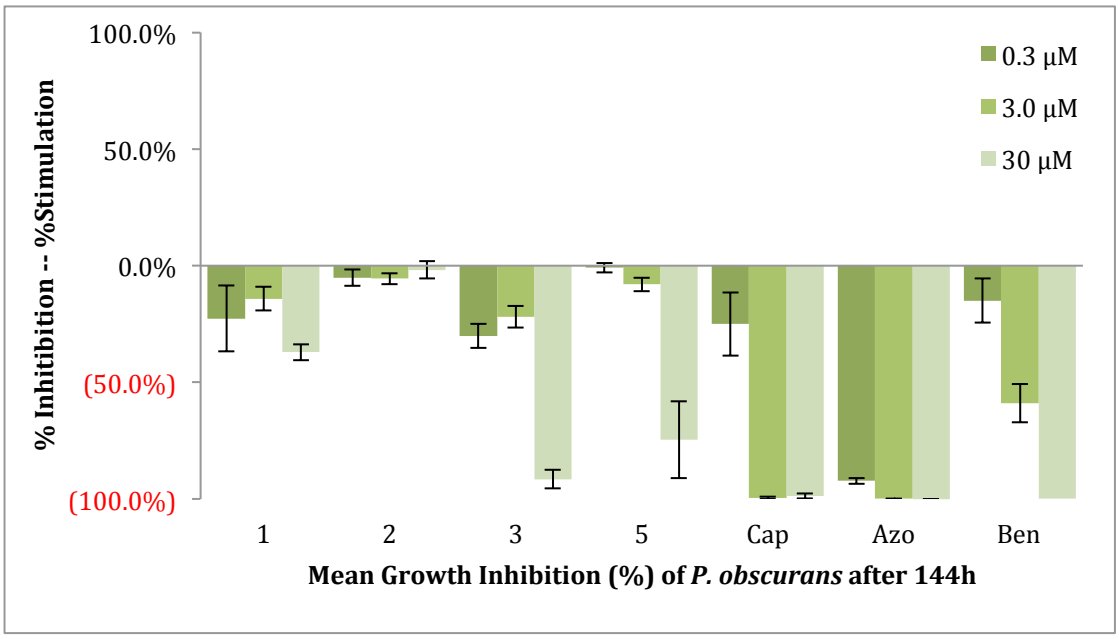


Figure 2.10 Mean fungal growth inhibition (%) of *Phomopsis obscurans* after exposure to 1, 2, 3 and 5 using a dose-response format at 120 and 144 h was noted**.** Abreviations used are: Cap: Captan; Azo: Azoxystrobin; Ben: Benomyl. The bold numbers represent the compounds in Figure 2.6. (**1**: Compound **9**; **2**: Compound **10**; **3**: Compound **11**; **5**: Compound **13**.

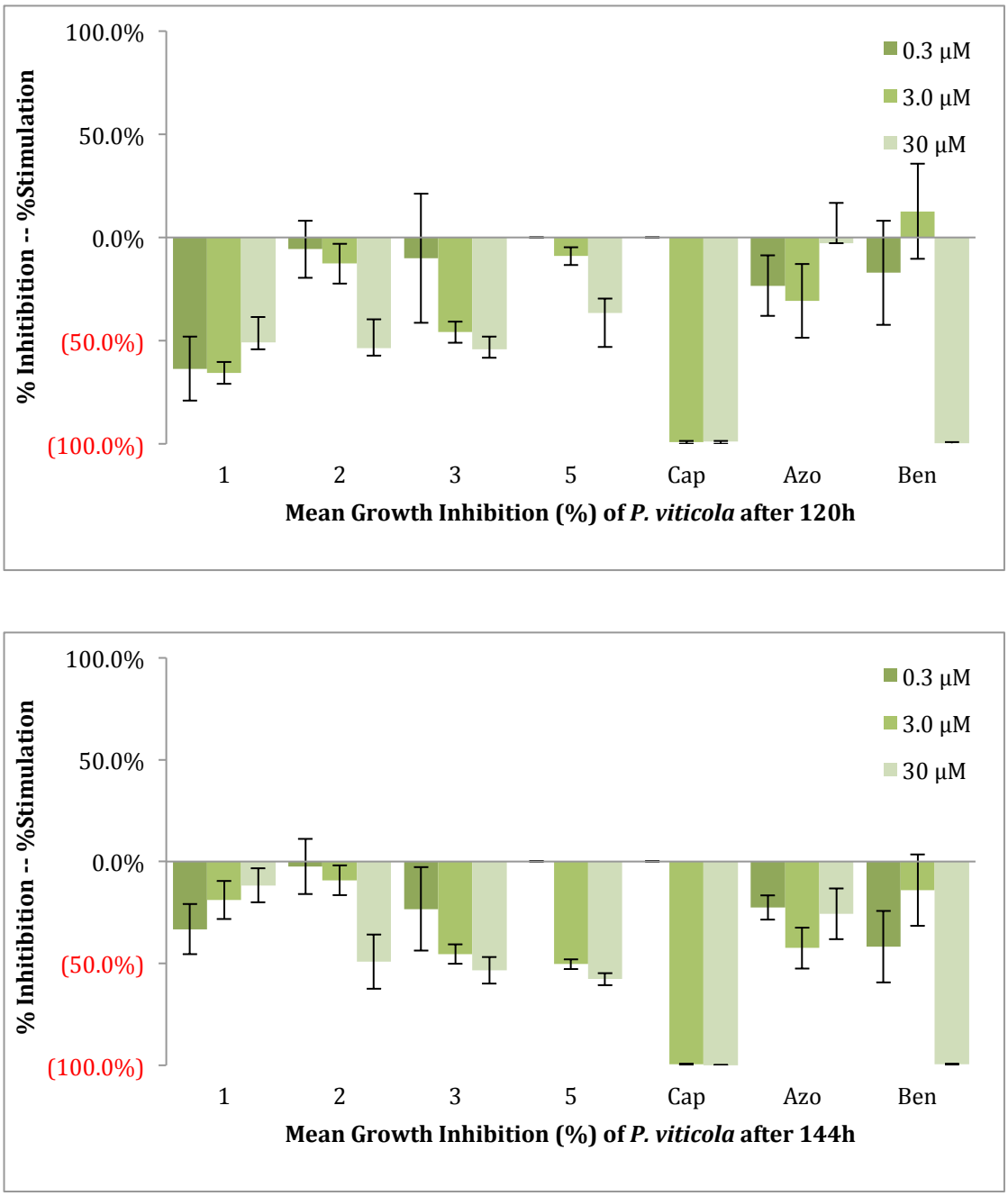


Figure 2.11 Mean fungal growth inhibition (%) of *Phomopsis viticola* after exposure to 1, 2, 3 and 5 using a dose-response format at 120 and 144 h was noted.

2.5 CONCLUSION

Using a bioassay-guided fractionation of the acetone extract *D. virginiana*, we isolated the antifungal constituents of the roots of this plant. This research demonstrates that OPLC is a powerful technique that can be used to separate and isolate co-migrating natural products produced by plants. These constituents were tested for the first time against *Colletotrichum* spp., *B. cinerea*, *F. oxysporum*, *P. obscurans*, and *P. viticola*. The most promising agricultural lead compounds against the pathogen *P. obscurans* are compounds **11** and **13**. Phomopsis leaf blight and fruit rot is a serious disease with strawberries and causes serious economic loss of this fruit each year. Our results suggest that compounds **11** and **13** warrant further *in vivo* testing as plant protectants or leads to control *Phomopsis* species [136].

CHAPTER 3 ISOLATION OF ANTIFUNGAL METABOLITES FROM TWO ISOLATES OF *CLADOSPORIUM CLADOSPORIOIDES*

3.1 INTRODUCTION

3.1.1 *Cladosporium* spp.

Cladosporium spp., one of the largest genera of dematiaceous hyphomycetes, is widely distributed throughout the world especially in the tropics and subtropics [140]. It contains more than 30 species and 772 names [141], and the most common species include *C. elatum*, *C. harbarum*, *C. spherospermum* and *C. cladosporioides* [142]. Species belong to *Cladosporium* are not characterized by common taxonomic classification due to their lack of sexual reproduction, and, hence are grouped into *Fungi imperfecti* [143], in which fungi produce their spores asexually. Identification and differentiation of the species of this genus are based on morphological and molecular methods. This genus is characterized by having a unique coronate scar structure [144], and by being linked to *Davidiella* teleomorphs [145, 146]. Colonies of *Cladosporium* were reported to have a unique color ranging from olive-green to greenish brown on PDA. They grow relatively slowly, but sporulate abundantly, giving the colonies a dusty, velvety appearance [147].

Cladosporium spp. is considered as the most isolated fungi in nature and can be found on dead plants, contaminated food, straw, soil, colors, and textile [143]. Cladosporium rot in wine

grapes was the common disease induced by two species, *C. cladosporioides* and *C. herbarum* [148]. Besides plants, *Cladosporium* spp. can also be pathogenic and toxigenic to humans. So far, research literatures have recorded cases of cerebral phaeohyphomycosis, cutaneous infections, onychomycosis, sinusitis and pulmonary infections caused by *Cladosporium*spp [143]. In addition, spores of this genus are easily spread in air and cause serious allergic diseases to respiratory tract mucosa or asthma as well as intrabronchial lesions [149, 150].

3.1.2 *Cladosporium cladosporioides*

Cladosporium cladosporioides (Fresen.) de Vries is a very common saprophytic fungus in this genus which can contaminate many types of seeds. It is the pathogen of many different host plants and can be isolated from many sources including air, soil, textiles and several other substrates [151]. In a direct-bioautography assay, *C. cladosporioides* are usually one of the adopted indicator organisms used for the detection of antifungal fractions and compounds in the bioassay-guided isolation [152, 153, 154, 155]. *C. cladosporioides* has also been involved in several human diseases causing pulmonary constrictions (asthma and pulmonary emphysema) and cutaneous infections (phaeohyphoomycosis) [149, 156, 157, 158]. In 2002, *C. cladosporioides* was reported to exist in three cerebrospinal fluid specimens and a brain biopsy specimen of a human patient [159]. Currently, 34 strains of *C. cladosporioides* have been isolated and identified from different sources [140]. A strain refers to a genetic variant of the same species. So one fungus could have a variety of strains under the same species name. Plant extracts have been considered to be ideal candidates for treating fungal infections. However, besides plant extracts natural occurring microorganisms can also be useful in the control of plant pathogens, such as *Penicillium* and *Trichoderma*. Recently, one invention reported that an isolate of *C. cladosporioides* was very effective in both prevention and treatment of pathogen caused diseases on plant issues, such as

apple scab (*Venturia inaequalis*) [160]. A particular isolate of *C. cladosporioides* combined with benzoic acid was effective against *Plasmopara viticola* by 81.27% growth inhibition [161]. An isolate has been used for fungus infections caused by *Botrytis* and *Fusarium* species [162]. More interestingly, *C. cladosporioides* has been widely used for biosorption of gold or silver from solutions[163]. A fungal isolate *C. cladosporioides* 1 was found to possess preferential metal binding properties and could efficiently adsorb gold and silver metals from a mixture which contain other metals including zinc, cadmium, chromium, nickel and copper [163]. Another isolate *C. cladosporioides* 2 could remove zinc, cadmium, chromium, nickel and copper in addition to gold and silver [164]. One report showed that *C. cladosporioides* was employed as reductant in the extracellular biosynthesis of silver nanoparticles (AgNP) [165] (Figure 3.1).

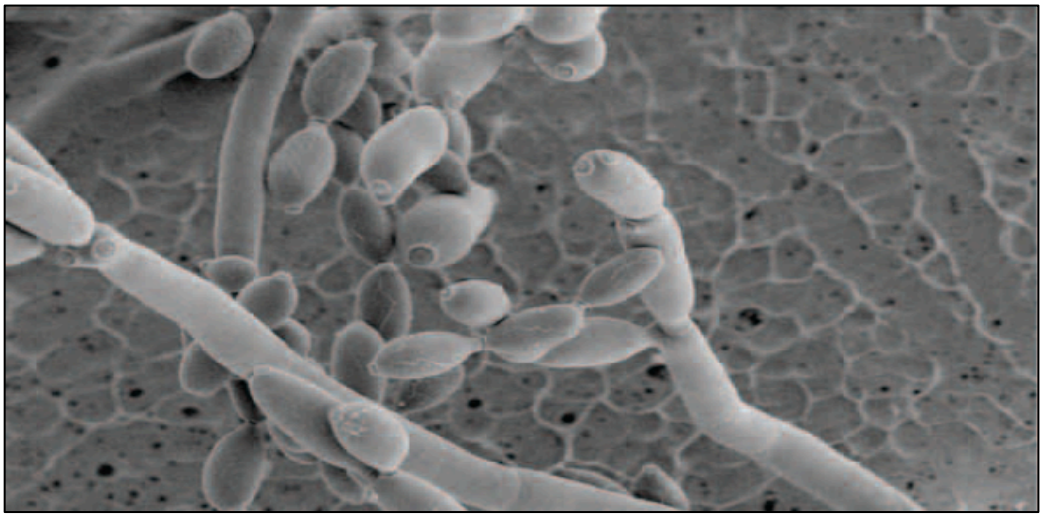


Figure 3.1 Conidiophores of *Cladosporium cladosporioides* sprouting from agar with all types of dispersion structures[140].

The pathogenicity and toxicity that *C. cladosporioides* produces to plants or human are normally attributed to the toxic secondary metabolites they produce. *C. cladosporioides* produces very active compounds, such as cladosporin and emodin, as well as some other less toxic

metabolites. Fungal secondary metabolites are bioactive compounds that are produced as families of related compounds during the stationary phase of growth and are usually of low molecular weight. Some famous fungal secondary metabolites are known as penicillin, cephalosporin, ergotrate and statins [166, 167]. Secondary metabolites do not appear to participate directly in fungal growth and development, but are involved in the interactions between fungi and their environment. In contrast, primary metabolites, such as vitamins, phytosterols, acyl lipids, nucleotides, amino acids, and alcohols are directly involved in normal fungal growth, development, and reproduction. In addition, primary and secondary metabolites have similar precursor molecules, chemical structures and biosynthetic origins [168]. Bioactive secondary metabolites that have been isolated from *C. cladosporioides* include cladosporin (**20**) [169], isocladosporin (**21**) [170], cladospolides A (**22**) and B (**23**) [171], cladosporol (**24**) [172] and the calphostins (**25**) [173] (Figure 3.2).

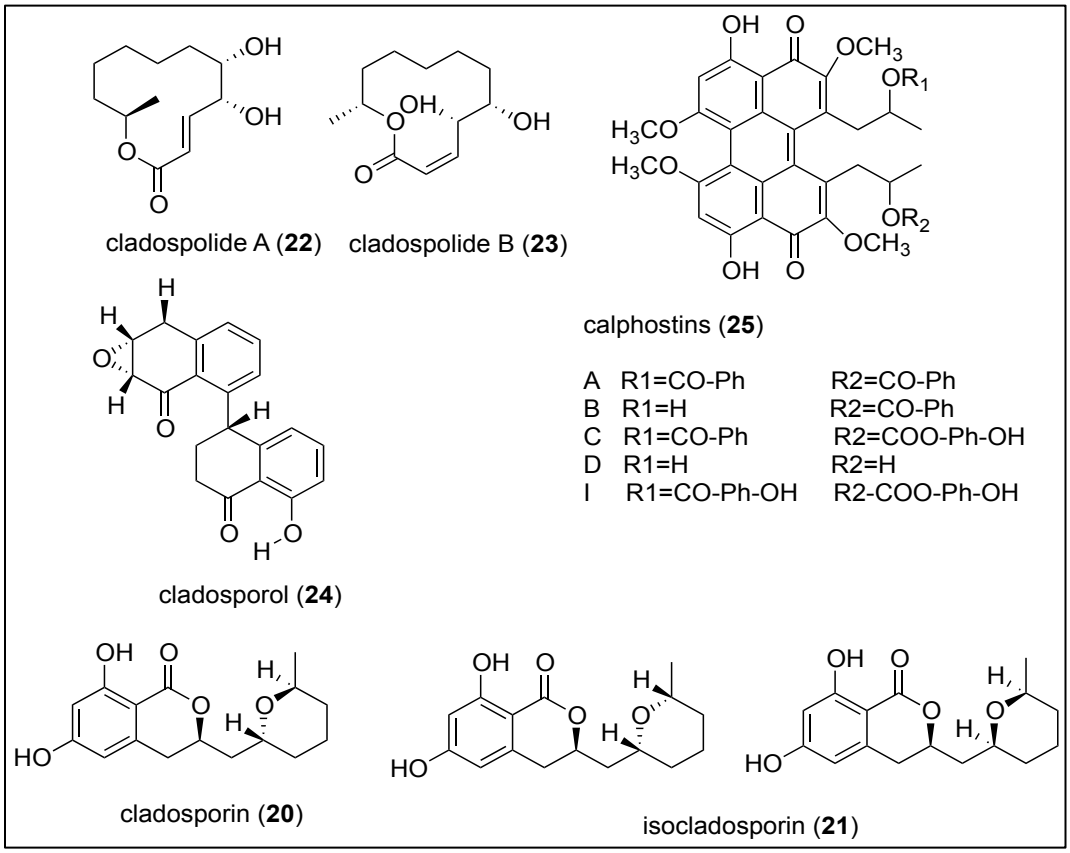


Figure 3.2 Structures of compounds isolated from *C. cladosporioides* by previous studies**.**

3.1.3 Biological and Chemical Study of Cladosporin

3.1.3.1 Biological activities of cladosporin

Cladosporin (**20**) (also known as asperentin), 3,4-dihydro-6, 8-dihydroxy-3-(6-methyl tetrahydropyran-2-ylmethyl) isocoumarin, is an important secondary metabolite derived from *C. cladosporioides* in 1971. It is a major compound of *C. cladosporioides* but a minor metabolite of other fungal sources including *Aspergillus flavus* [174], *Aspergillus repens* [175, 176], *Oidiodendron truncata* [177], *Chaetomium globosum* [178], and several *Eurotium* species [176]. It showed specific antifungal and antibacterial properties, as well as insecticidal activity [170, 179]. It was reported to inhibit the spore generation of *Penicillum* and *Aspergillus* species at ≤ 40 μg/mL, and uracil and

leucine uptake into *Bacillus brevis* cells [171]. Also, it completely inhibited the growth of several dermatophytes *in vitro* at 75 μg/mL[169]. Xie, *et al.*, found that cladosporin exhibited antifungal activity against *Escherichia coli*, *Rhizoctonia solani*, *Trichoderma viride* and *Colletotrichum cameliaeet al*. at a concentration of 100-200 μg/mL [180]. Cladosporin was shown to be a plant growth inhibitor by Springer and Cutler in 1981, which inhibited the growth of etiolated wheat coleoptiles at 10^{-3}, 10^{-4}, and 10^{-5} M [175]. Later in 1994, cladosporin was tested for antitumor induction in an *Agrobacterium tumefaciens* potato disc assay and it showed -79% antitumor induction [181]. Due to these statistics, cladosporin has been considered as a promising novel fungicide.

3.1.3.2 Stereochemistry of cladosporin

Cladosporin was crystallized from methanol as pale yellow crystals and soluble in ethanol, and ethyl acetate, moderately soluble in chloroform, slightly soluble in benzene, and insoluble in hexane and water [169, 182]. Its stereochemistry was completely assigned through X-ray diffraction by Vederas, Cutler and other researchers at Merck (Figure 3.3) [175, 183].

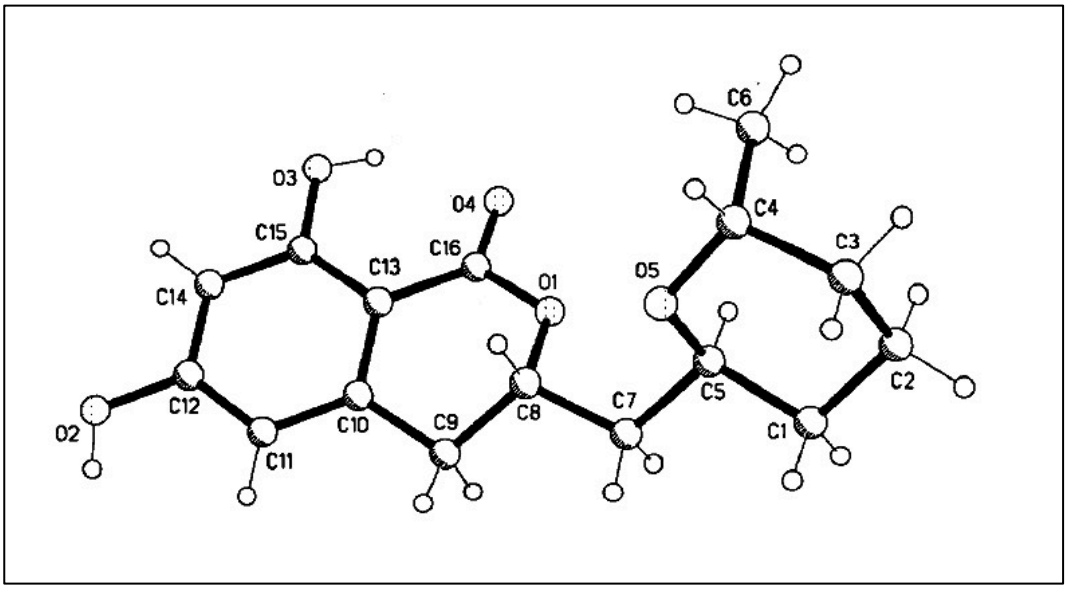


Figure 3.3 The crystalline structure of cladosporin**.**

As Figure 3.3 showed, the absolute configuration of three chiral carbons, C8, C4 and C5, need to be established. The stereochemistry of C8 was determined as *R* by circular dichroism (CD) analysis, which showed an opposite curve to that given by a known compound [174]. Subsequently, the X-ray structure assigned the stereochemistry of the tetrahydropyran ring at C4 (S) and C5 (R) [175]. The X-ray data was described in page 72.

3.1.3.3 Biosynthesis of cladosporin

Biosynthesis of cladosporin involves assembly of basic two carbon units from acetate and malonate through acetate-malonate (AA-MA) pathway, which is an important pathway in the biosynthesis of polyketides and fatty acids [184]. The formation of polyketides closely resembles the biosynthesis of long chain fatty acids [185]. This pathway involves a series of Claisen reactions in which two molecules of acetyl-CoA react in a Claisen condensation to give acetoacetyl-CoA, and then repeated to generate a poly-β-keto ester of required chain length (Figure 3.4).

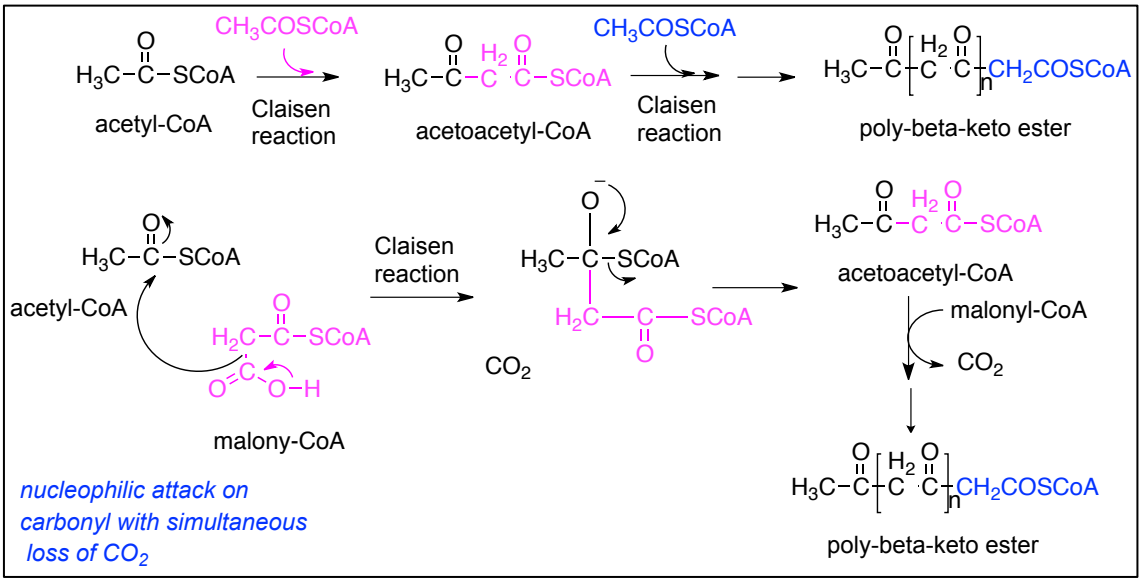


Figure 3.4 The formation of the poly-β-keto chain by claisen condensation**.**

In 1973, Grove and the co-workers reported the biosynthesis of cladosporin (II, Figure 3.5) in an entomogenous strain of *Aspergillus flavus* by incorporation of [1-^{13}C] acetate and [2-^{14}C] malonte [186]. This labeling experiment confirmed the biosynthetic pattern of cladosporin from a precursor (I, Figure 3.5) consisting of one acetate-derived initiating unit and seven malonate-derived two-carbon building units (Figure 3.5).

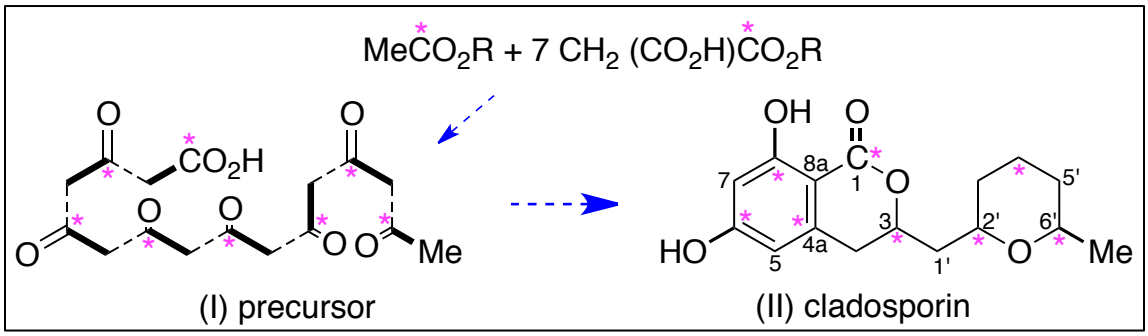


Figure 3.5 Biosynthesis of cladosporin ring system from acetate and malonate units**.**

3.1.3.4 Total synthesis of cladosporin

Recently, the asymmetric total synthesis of cladosporin was just reported by Chinese scientists in Lanzou University [187]. They started the synthesis in 8 steps from tetrahydropyran (THP), which was found in numerous biologically active natural products. Cladosporin (**20**) bears a 2, 6-disubstituted tetrahydropyran (THP) ring and a δ-valero lactone with a fused 1, 3-dihydroxybenzene ring. The synthesis started with **28**, which was generated from ring-open of oxide **27**. Treatment of allytrime-thylsilane in the presence of a catalytic amount of TMSOTf led to **29**, which had 2,6-*trans*-THP selectivity. Enantioenriched epoxide **30** was obtained by *m*-CPBA epoxidation of alkene **29**. After treatment with the Grignard reagent and CuI at -30 °C, epoxide **30** was reduced to alcohol *trans*-**4**. The lactone **32** was furnished from *trans*-**4** through two reactions including oxa-Pictet-Spengler cyclization and Jones oxidation. After removal of both aromatic methyl ether groups under Maier's conditions, lactone **32** was converted to cladosporin (Figure 3.6).

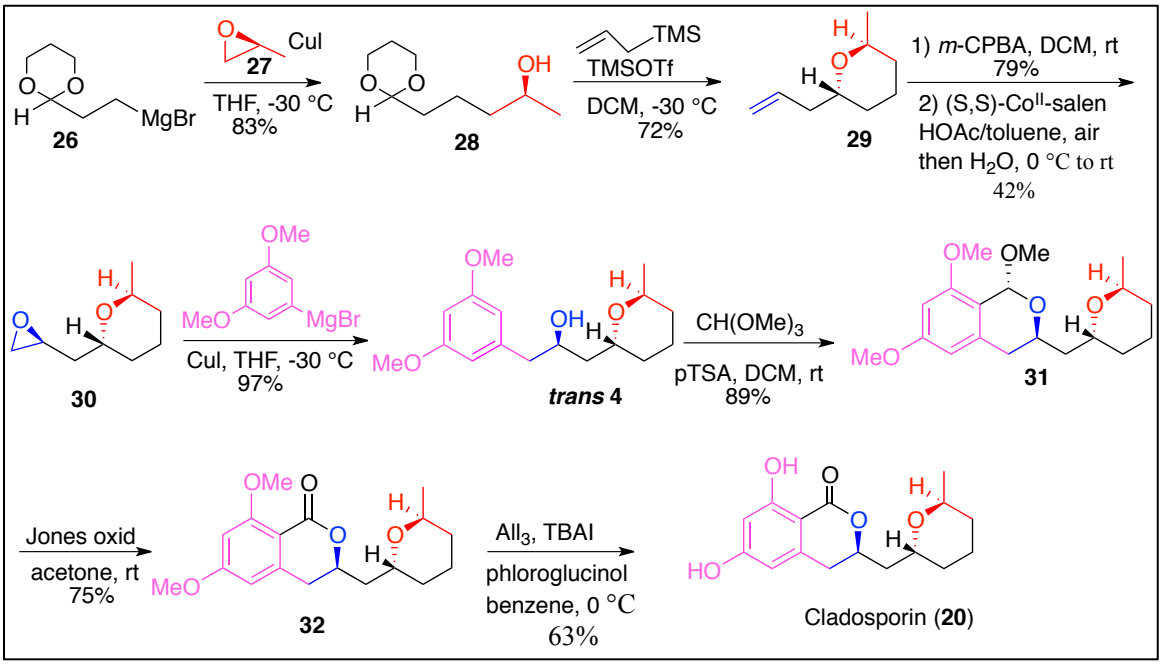


Figure 3.6 Total synthesis of cladosporin [187].

3.1.4 Biological and Chemical Study of Isocladosporin

Isocladosporin (**21**) is a diastereoisomer of cladosporin at C-14 (or C-6'), and was isolated from *C. cladosporioides* in 1993 [188]. The absolute configuration of natural isocladosporin has not been deduced. There exist two solution-state conformations (see Figure 3.7) that were proposed by Jacyno in 1993 [188]. In 2012, She, *et al*., synthesized the isocladosporin and proved that the relative configuration of isocladosporin was supposed to be the structure of (21. a) (Figure 3.7), because the physical properties of (21. b) was different from that of natural isocladosporin isolated by Jacyno [187]. Isocladosporin was tested to be slightly more potent than cladosporin in the etiolated wheat coleoptile bioassay, producing 100% growth inhibition, compared to 81% for cladosporin at 10^{-3} M [188].

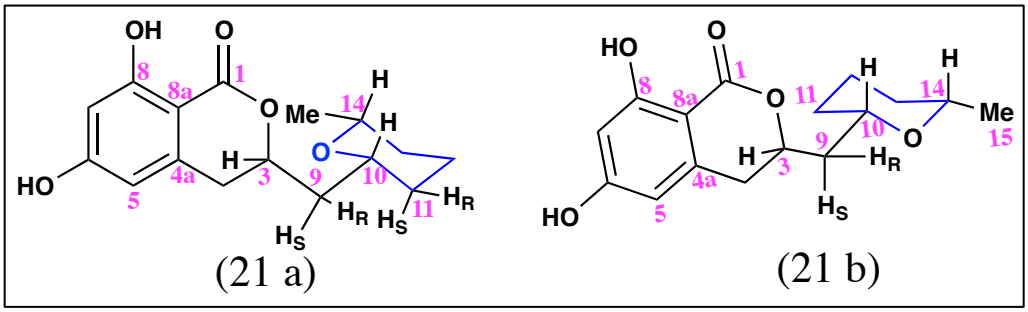


Figure 3.7 Two solution-state conformations (a & b) of isocladosporin. In structure b, H-11 protons have been omitted for clarity.

3.1.5 Other Compounds Isolated From *C. cladosporioides*

Other compounds isolated from *C. cladosporioides* include cladospolides A (**22**) and B (**23**), cladosporol (**24**), and the calphostins (**25**). Cladospolides A (**22**) and B (**23**) are two geometrical isomers that were isolated by Hirota and co-workers in 1981 and 1985 respectively. It is interesting to know that both isomers showed different biological activities--cladospolides A acted as a root growth inhibitor of lettuce seedlings, while cladospolides B acted as a root growth

promoter of lettuce seedlings [189, 190]. In 1995, one more bioactive metabolite was isolated from *C. cladosporioides* elucidated as cladosporol (**24**), which was a β-l, 3-glucan-biosynthesis inhibitor due to its epoxy-alcohol structure [172]. A complex of Calphostins (**25**) (A, B, C, D, I) was isolated from *C. cladosporioides* by Japanese scientists in 1989. These calphostins showed potent and specific inhibitory activity against protein kinase C (PKC), with emphasis of calphostin C that specifically inhibited PKC at 0.05 μM [173].

3.2 THE AIM OF THIS RESEARCH

In summary, fungi provide an abundant source of natural products that may have potential agricultural, environmental, and pharmaceutical use. In the course of discovery of bioactive metabolites from fungus extracts, 40 fungi crude extracts were screened using a direct-bioautography coupled *Colletotrichum* bioassay. Eight extracts were identified as active. Two of the extracts, the acetone extracts of *Cladosporium cladosporioides* NRRL 5507 and another isolate of *C. cladosporioides* (labeled as Miligrante) showed the most promising activities against three *Colletotrichum* species (Figure 3.8). To our best knowledge, there are no reports on the antifungal activity of *C. cladosporioides* against the three *Colletotrichum* species. The crude extracts, fractions, and pure compounds of *C. cladosporioides* were also tested for cannabinoid and opioid receptor activity. Based on the encouraging biological effects, the isolation and purification of the crude extracts of this species was initiated.

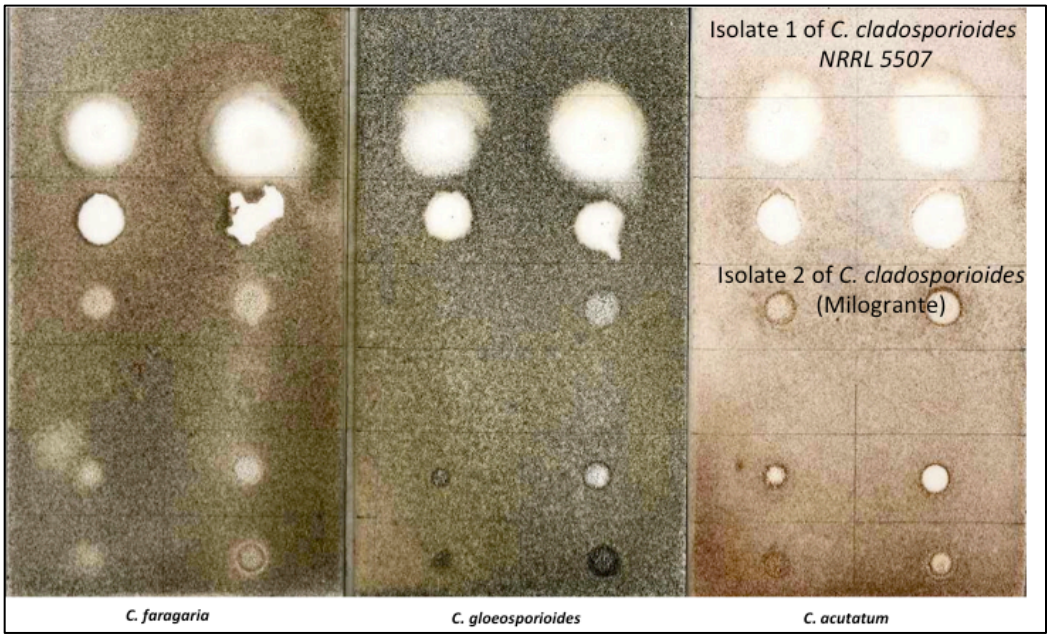


Figure 3.8 Bioautographic results of two isolates of *C. cladosporioides* against three *Colletotrichum* species. The first row showed the result of isolate 1 (*C. cladosporioides* NRRL 5507) and the second row showed the result of isolate 2 (Milogrante).

3.3 BIOLOGICALLY ACTIVE COMPOUNDS FROM *CLADOSPORIUM CLADOSPORIOIDES* NRRL 5507

3.3.1 Results and Discussion

Bioassay-guided fractionation of *C. cladosporioides* crude extracts led to the isolation of six compounds, including the major constituent cladosporin (**20**) (5.038 g), isocladosporin (**21**) (8.3 mg), 5'-hydroxyasperentin (**33**) (117 mg), asperentin-8-methyl ether (**34**) (3 mg), palmitic acid (**36**) (10 mg) and linoleic acid (8 mg) (**37**). And the compound, 6, 5'-diacetyl cladosporin (**35**) (4.5 mg) was synthesized based on pyridine-catalyzed acetylation of compound **33** (Figure 3.9). The chemical structures of these compounds were established on the basis of extensive 1D and 2D NMR and HRESIMS data. Cladosporin, as a major component, was crystallized from

three fractions, Hex/EtOAc (25:75), Hex/EtOAc (50:50), and EtOAc(100%) fractions as pale yellow crystals. The absolute stereochemistry of cladosporin was validated by single-crystal X-ray. Compounds **33** and **34** were isolated from *Aspergillus flavus* [174], *Chaetomium globosum* [178] and *Eurotium repens* before [179] but they were isolated for the first time from *C. cladosporioides*. The stereochemistry of 5'-hydroxyasperentin (**33**) at C-5' was confirmed for the first time on the basis of NOE data. Pure compounds were submitted for antifungal activities against seven plant pathogens. *Phomopsis viticola* was the most sensitive fungus to the test compounds. *Phomopsis viticola* is a plant pathogen that causes severe disease of grape often known as Phomopsis cane and leaf spot disease of grape**.** At 30 μM, Cladosporin (**20**) exhibited 92.7%, 90.1%, 95.4% and 83.0% growth inhibition against *C. acutatum*, *C. fragariae*, *C. gloeosporoides* and *P. viticola*, respectively. Isocladosporin (**21**) showed 50.4%, 60.2% and 83.3% growth inhibition at 30 μM against *C. fragariae*, *C. gloeosporoides* and *P. viticola*, respectively. Cladosporin exhibited antifungal activity against *Cryptococcus neoformans* with IC_{50} value of 17.72 μg/mL. Furthermore, in herbicidal bioassays, cladosporin has moderate to high monocot herbicide activity against Agrostis but no dicots herbicide effect against Lettuce.

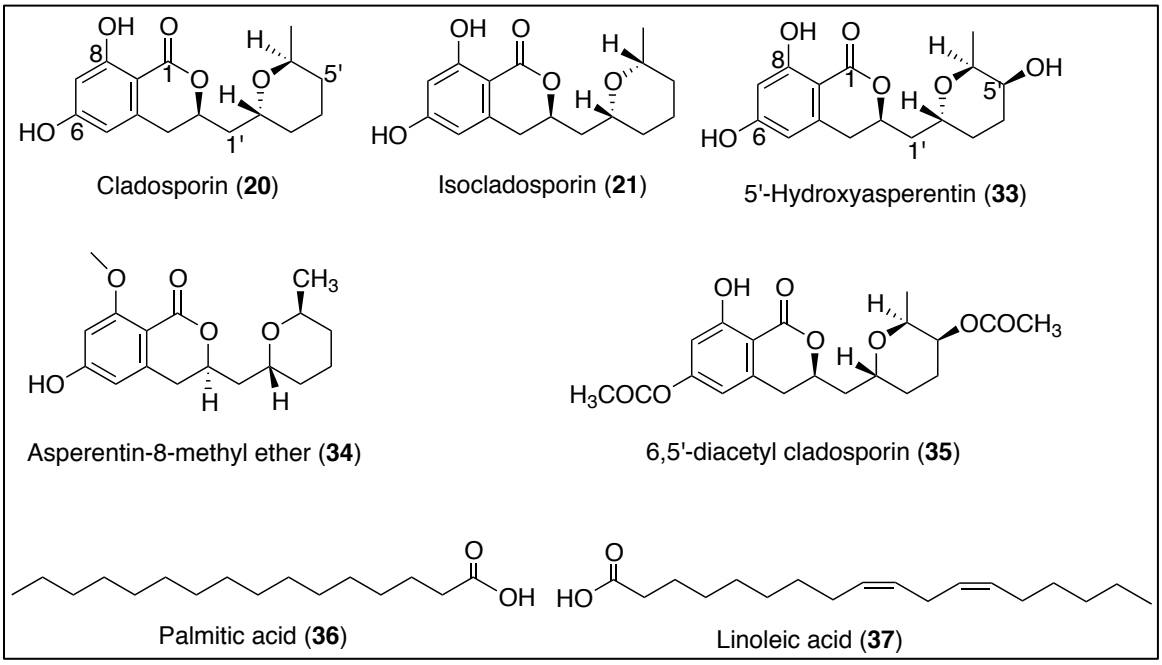


Figure 3.9 Compounds isolated from *Cladosporium cladosporioides* NRRL 5507**.**

3.3.2 Biological Evaluation

3.3.2.1 Antifungal activity against plant pathogens (Microtiter Assay)

Pure compounds were further evaluated for their antifungal activity using an *in vitro* micro-dilution broth assay. Compound **20** showed significant antifungal activities and at 30 µM caused 92.7 % growth inhibition of *C. acutatum*, 90.1 % of *C. fragariae,* 95.4% of *C. gloeosporoides* at 48 h, and showed promising antifungal selectivity against *P. viticola* (79.9 %) and *P. obscurans* (-21.7%) at 120 h. (Figure 3.9-3.13). In Figure 3.9 and 10, compound **20** exhibited moderate antifungal activities against *C. acutatum* and *C. fragariae* compared to the standard fungicide azoxystrobin, which only caused 40.5% and 58.9% respectively. These data suggested that compound **20** could be considered as a novel natural product lead for the development of fungicidal agent against strawberry pathogens. Compound **21** showed low antifungal activity and

at 30 µM caused 50.4% growth inhibition of *C. fragariae*, 60.2% of *C. gloeosporoides* at 48 h (Figure 3.10 and 3.11). Compound **21** showed promising antifungal selectivity between *P. viticola* (80.3 %) and *P. obscurans* (22.5%) at 120 h (Figure 3.12 and 3.13). Compound **26** and **28** showed no antifungal activities against three *Colletotrichum* species, but showed good selectivity over *P. viticola* (53.9% and 79.4%) and *P. obscurans* (25.6% and 10.3%) (Figure 3.12 and 3.13). Due to these properties, compounds **20**, **21**, **33** and **35** could be further evaluated as fungicidal leads for pest and disease control.

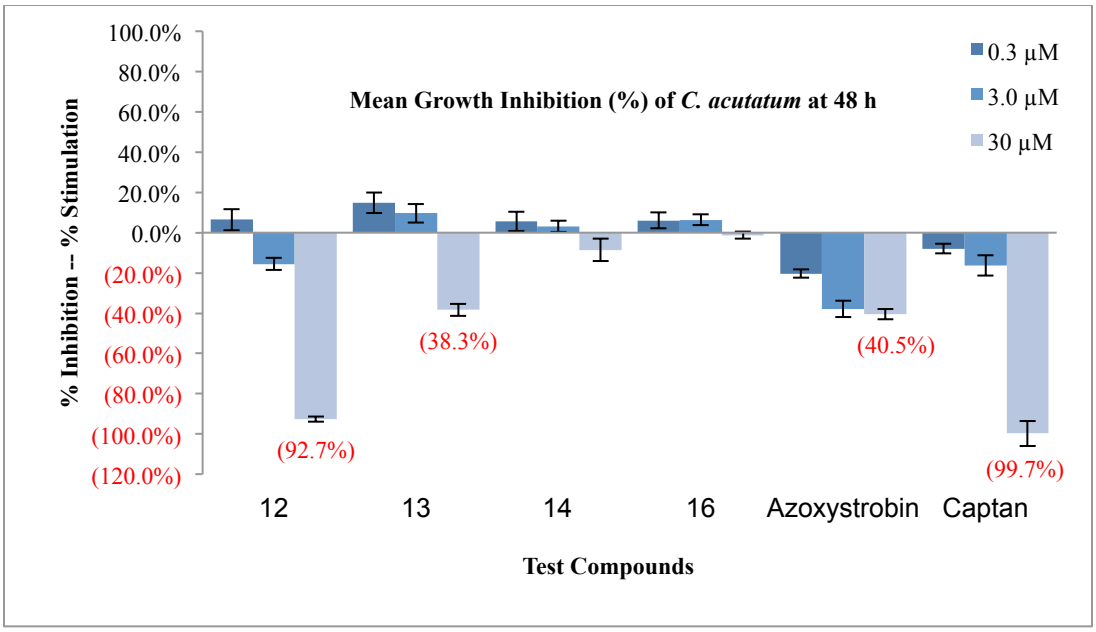


Figure 3.10 Mean fungal growth inhibition (%) of *Colletotrichum acutatum* after exposure to 12, 13, 14 and 16 using a dose-response format at 48 h. Abreviations: Cap: Captan; Azo: Azoxystrobin; Ben: Benomyl. (**12**: compound **20**; **13**: compound **21**; **14**: compound **33**; **16**: compound **35**).

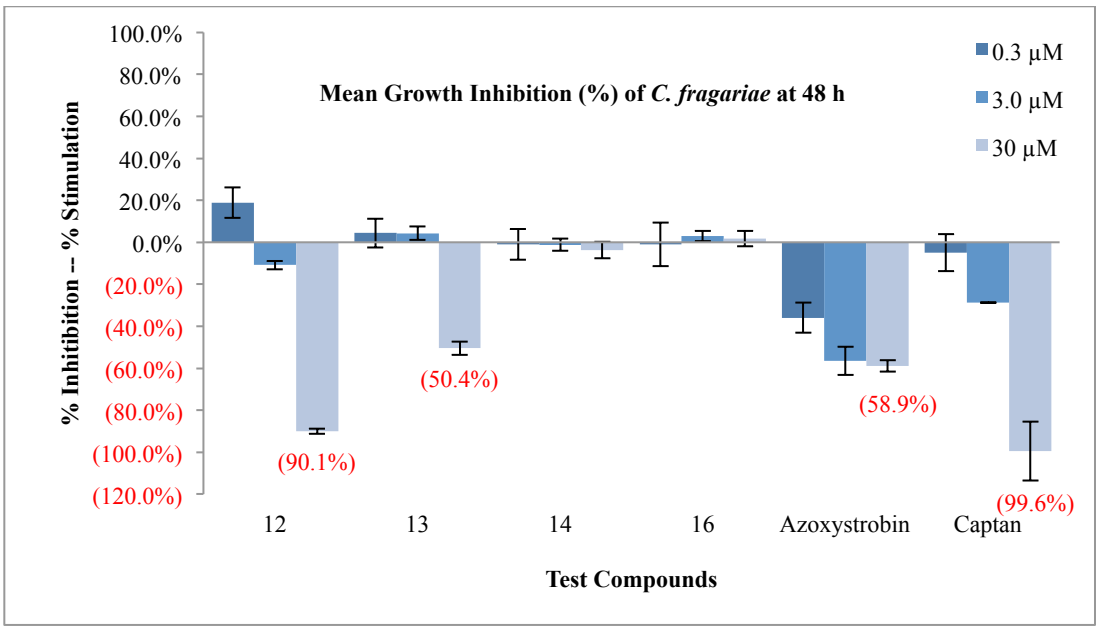


Figure 3.11 Mean fungal growth inhibition (%) of *Colletotrichum fragariae* after exposure to 12, 13, 14 and 16 using a dose-response format at 48 h**.**

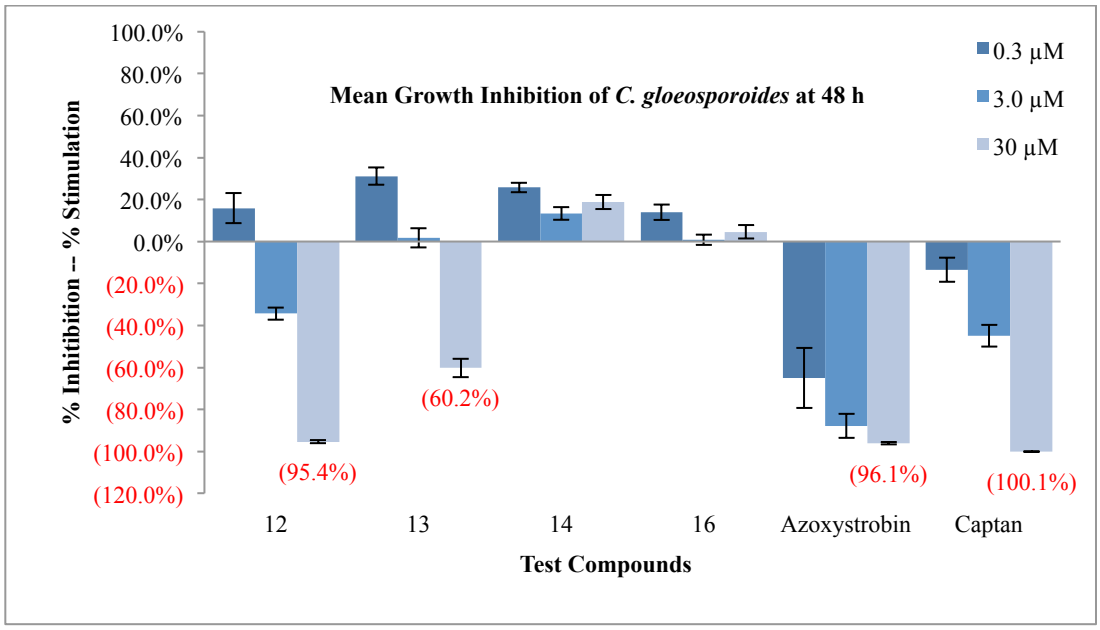


Figure 3.12 Mean fungal growth inhibition (%) of *Colletotrichum gloeosporoides* after exposure to 12, 13, 14 and 16 using a dose-response format at 48 h**.**

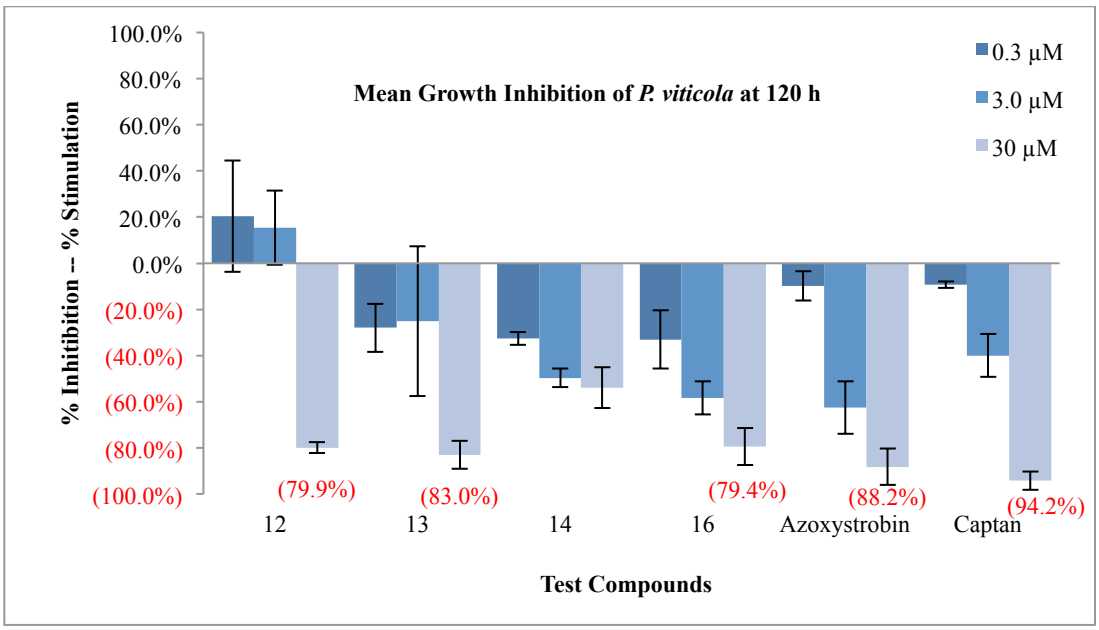


Figure 3.13 Mean fungal growth inhibition (%) of *Phomopsis viticola* after exposure to 12, 13, 14 and 16 using a dose-response format at 120 h.

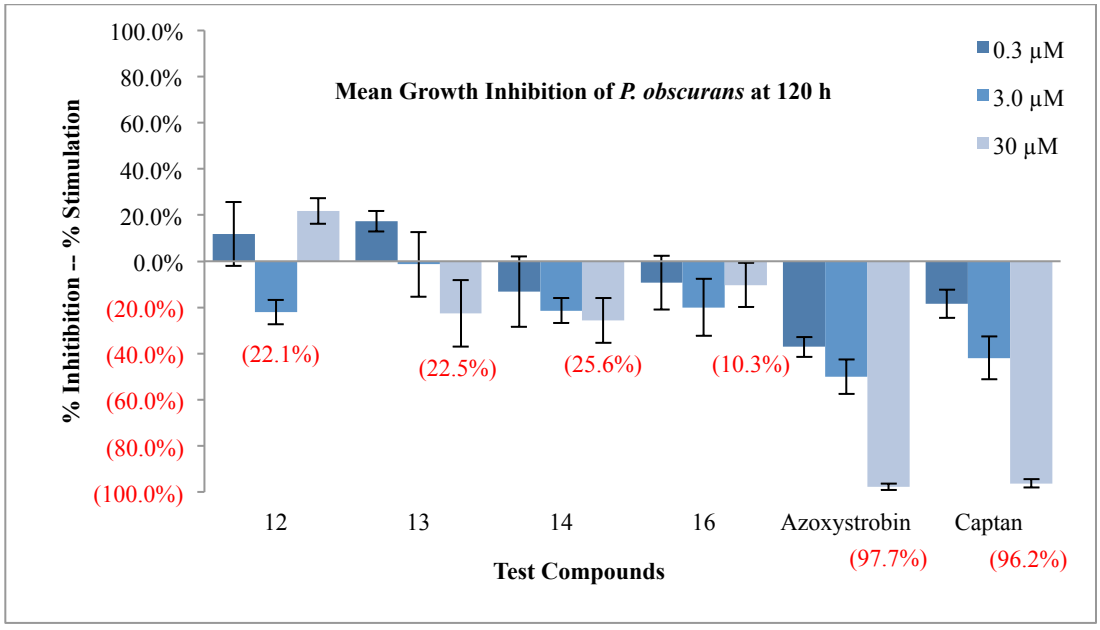
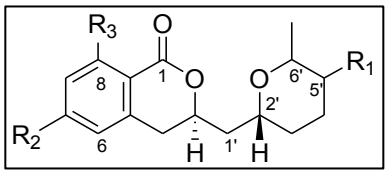


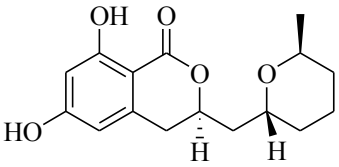
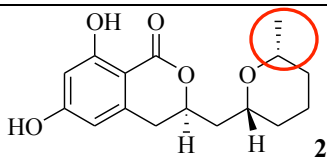
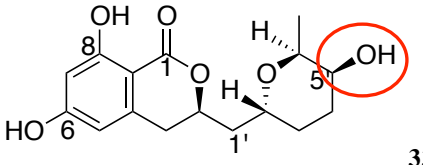
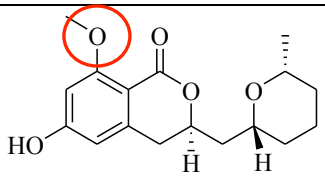
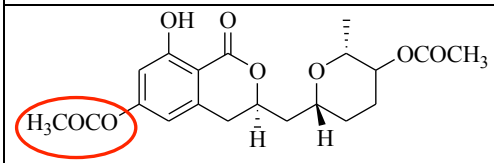
Figure 3.14 Mean fungal growth inhibition (%) of *Phomopsis obscurans* after exposure to 12, 13, 14 and 16 using a dose-response format at 120 h.

After overall study of the relationship between the structures and antifungal activities of the pure isolates at 30 μM, we found several essential positions that might be responsible for their antifungal activities (Table 3.1). Comparing the structures between compound **20** and **21**, the absolute configuration of C-6' affects antifungal activity. R configuration of C-6' in **21** greatly decreased antifungal activity against *Colletotrichum* species, but slightly increased the antifungal activities against *Phomopsis species*. Comparing the structures between **21** and **33**, introduction of one hydroxyl group at C-5' position caused complete loss of antifungal activity against *Colletotrichum* species and decreased the selectivity against *Phomopsis* species, which indicated the importance of openness of 5' position to keep it active. By comparing the structures between **21** and **34**, the replacement of hydroxyl group with methoxy group at C-8 caused broad loss of antifungal activities against all the test fungi, which indicated this position might be the active site where hydrogen bonds were formed to enhance the interaction between the enzyme and agent. Comparing compound **33** and **35**, the replacement of hydrogen at C-6 and C-5'with acetyl groups greatly increased the selectivity against *Phomopsis* species.

Therefore, S configuration of C-6', openness of C-5', hydroxyl group at C-8, and introduction of functional groups at C-6 are several remarkable observations we obtained from the antifungal SAR study. This might provide us some useful information for further SAR study.

Table 3.1 Overall Fungal Growth Inhibition of Compounds **20**, **21** and **26-30** Against Plant Pathogens at 30 μM.



Chemical Structures	*C. fraga.*	*C. gleos.*	*C. acut.*	*P. obscu*	*P. vitico.*
 **20**	**90.1%**	**95.4%**	**92.7 %**	NA	**79.9%**
 **21**	**50.4 %**	**60.2 %**	38.3 %	22.5%	**83.0%**
 **33**	NA	NA	NA	25.6%	**53.9%**
 **34**	NA	NA	NA	NA	35.1%
 **35**	NA	NA	NA	10.3%	**79.4%**

3.3.2.2 Opioid &cannabinoid receptor binding affinity

The crude extracts, fractions and pure compounds isolated from this species have been submitted for testing to determine their affinity for opioid receptors (subtype δ, κ and μ) and cannabinoid receptors (subtype CB1 and CB2). The result showed that only the crude extracts possessed modest opioid effect (δ, 70.11; κ, 65.67; μ, 56.58). The binding affinity was lost in the sub-factions and pure compounds of *C. cladosporioides* (Table 3.2). This indicated that the combination of the active compounds in the crude extracts contributed to the affinity of opioid receptors other than single compound. Synergism refers that two or more agents, when used together, produce an effect greater than one single agent. It is very common phenomenon in fungal extracts in which the active compounds interact within themselves to synergize the therapeutic effects [191].

Table 3.2 Binding Affinity Assay of the *Cladosporium cladosporioides* for Human Opioid Receptors and Cannabinoid Receptors.

Species	Fractions (10 μg/mL)	Opioid Receptors (%)			Cannabinoid Receptors (%)	
		δ	κ	μ	CB1	CB2
Cladosporium cladosporioides	Crude extracts	70.11	65.67	56.58	21.6	20.5
	25% Hexane in EtOAc	53.7	-10.95	-27.96	36.3	8.3
	50% Hexane in EtOAc	36.5	-43.54	-12.89	0.9	10.0
	EtOAc	27.2	-24.44	-37.72	-16.1	4.8
	25%MeOH in EtOAc	27.4	-14.57	-41.96	26.4	5.5
	MeOH	37.2	-20.49	-62.40	17.1	3.9
	Cladosporin (10 μM)	-39.7	-24.1	42.3		
	5'-Hydroxyasperentin (10 μM)	-0.2	-18.9	-6.2		
Naloxone (10 μM)		101.3	103.8	99.7	-	-
CP 55,940 (10 μM)		-	-	-	99.4	101.0

Note: For crude extracts and their fractions, the concentration is 10 μg / mL; for pure isolates, 10 μM was used.

3.3.2.3 *In vitro* antimicrobial activities of cladosporin and 5'-hydroxyasperentin

Since we have a good quantity of cladosporin (5.038 g) and 5'-hydroxyasperentin (117 mg) (Figure 3.9) (the quality of other pure compounds is less than 1 mg and not enough for

further studies), these two compounds were submitted for their antibacterial and antifungal (against human pathogens) activities. The antibacterial activities were tested against *Staphylococcus aureus*, methicillin-resistant *S. aureus* (MRS), *Escherichia coli*, *Pseudomonas aeruginosa*, and *Mycobacterium intracellulare*. Ciprofloxacin was used as positive control for antibacterial activity.

The antifungal activities were evaluated against a panel of pathogenic fungi including *Candida albicans*, *C. glabrata*, *C. krusei*, *Aspergillus fumigatus* and *Cryptococcus neoformans* associated with opportunistic infections. Amphotericin B was included as a standard antifungal drug for comparison. Cladosporin exhibited antifungal activity against *Cryptococcus neoformans* with IC_{50} value of 17.72 μg/mL but no antibacterial activity against test organisms (if compounds are not active at the highest test concentration of 20 μg/mL, we considered it to be inactive). 5'-hydroxyasperentin did not show any antibacterial activity or antifungal activity against the ten species. This indicated that substituents at 5'-position might lead to the loss of antifungal activity. Furthermore, the number of double bonds in the side chain of the structure is normally crucial for maintaining both antibacterial and antifungal activity. Both compounds lack of long side chain with double bonds, which might be the reason for their weak or inactive properties.

3.3.2.4 Herbicidal activity of cladosporin

Cladosporin was also tested for its potential herbicidal activity. Three different concentrations (10, 100 and 1000 μM) were used. Ranking of plant growth was subjective. A ranking of 0 indicated no apparent inhibition (sample well plants looked identical to the Control + Solvent well plants). A ranking of 5 indicated no growth or complete inhibition. A ranking of five was given only if no seeds germinated. From Table 3.3, Cladosporin showed moderate to

high monocot herbicide activity against Agrostis, but no effect against dicots herbicidal activity against Lettuce. Monocots (or monocotyledons) normally have a single embryonic seed leaf, leaves with parallel veins, and flowers with parts in threes, while dicots (or dicotyledon) have two embryonic seed leaves and leaves with netlike veins.

Table 3.3 Herbicidal Activity of Cladosporin

Compound	Concentration	Solvent	Day	Ranking	
				Lettuce	Agrostis
Cladosporin	10 μM	10% acetone	7	0	0
Cladosporin	100 μM	10% acetone	7	0	1
Cladosporin	1000 μM	10% acetone	7	0	4

(Ranking based on scale of 0 to 5. 0=no effect; 5=no growth)

3.3.3 Experimental Sections

3.3.3.1 General experimental procedures

All melting points were obtained on a Thomas Hoover Uni-melt Capillary Melting Point Apparatus and are uncorrected. All IR spectra were generated neat on an ATI Mattson FT-IR using NaCl plates. Optical rotations were recorded using a Rudolph Research Analytical Autopol V polarimeter. UV was respectively obtained using a Perkin-Elmer Lambda 3B UV/vis spectrophotomer. ^{1}H and ^{13}C NMR spectra were obtained on Bruker model AMX 500 NMR spectrometers with standard pulse sequences, operating at 500 MHz in ^{1}H and 125 MHz in ^{13}C. The chemical shift values were reported in parts per million units (ppm) from trimethylsilane (TMS) using known solvent chemical shifts. Coupling constants were recorded in Hertz (Hz). Standard pulse sequences were used for COSY, HMQC, HMBC, TOCSY, NOESY and DEPT.

High-resolution mass spectra (HRMS) were measured on a Micromass Q-Tof Micro mass spectrometer with a lock spray source. Column chromatography was carried out on silicagel (70-230 mesh, Merck) and Sephadex LH-20 (Mitsubishi Kagaku, Tokyo, Japan). TLC (silica gel 60 F254) was used to monitor fractions from column chromatography. Preparative TLC was carried out on silica gel 60 PF 254+366 plates (20 × 20 cm, 1 mm thick). Visualization of the TLC plates was achieved with a UV lamp (λ=254 and 365 nm) and anisaldehyde/acid spray reagent (MeOH: acetic acid: anisaldehyde: sulfuric acid, 85:9:1:5). HPLC analyses were performed on a Waters LC Module I equipped with a UV detector 486 utilizaing the Millenium 32 Chromatography Manager software (Waters). An ODS column (Phenomenex Luna C18, 10 × 250 mm, 5 μm) was used. All HPLC solvents were HPLC grade, filtered through appropriate membranes (water through 0.45 μm and organic solvents through 0.22 μm filters) and sparged prior to and during analysis with nitrogen at a flow rate of 50 mL/min.

3.3.3.2 Fungal material

The fungus was collected in Tifton, Georgia, in 1978, lyophilized, and stored at -20 °C. The fungus was plated out on potato-dextrose agar, which was maintained at 24°C until discrete fungal colonies appeared. Then 50 mL of potato-dextrose broth was inoculated with the fungus and incubated for two weeks in stationary phase at 24 °C. The fungus was subsequently seeded onto a shredded wheat medium consisting of100 g of shredded wheat and 200 mL of Difco mycological broth (pH 4.8) (40 g of yeast extract, 400 g of sucrose and 100 g of mycological broth in 2 L DDI H_2O) in a 2.0 L Fernbach flask (15 flasks were used) followed by incubation for 22 days at 24 °C [188].

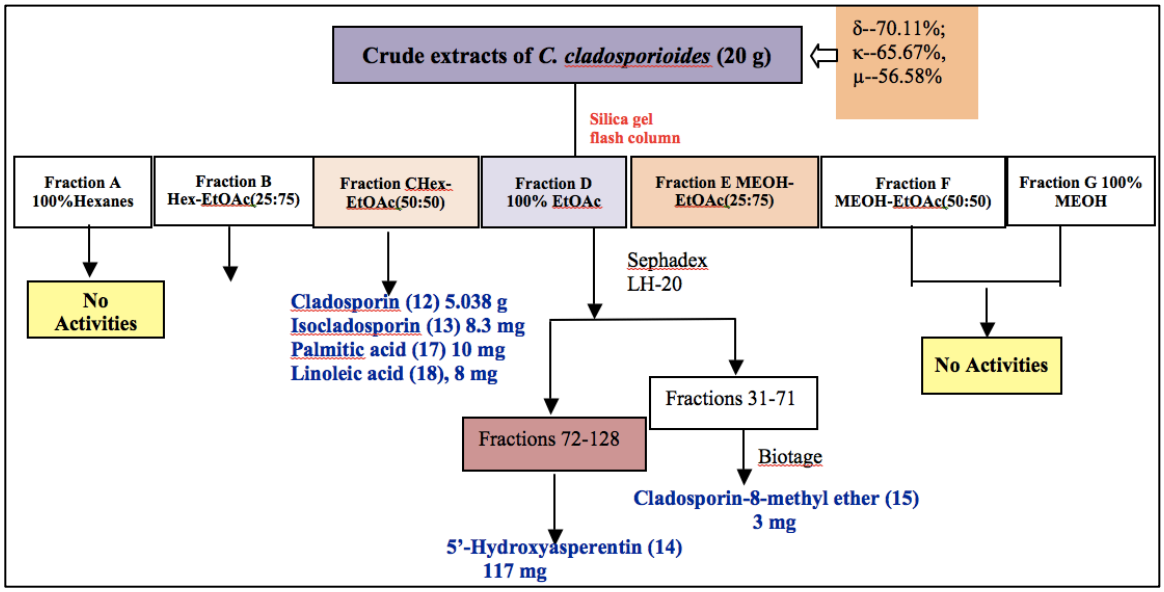


Figure 3.15 Bioassay-guided isolation of bioactive compounds from *C. cladosporioides* using bioautography.

3.3.3.3 Extraction and isolation

Following incubation, 300 mL of acetone was added to each flask, and the fungus and the substrate were homogenized. The suspension was filtered and the filtrate was concentrated under vacuum at 40 °C to yield water fraction. The water extraction was then extracted with EtOAc (500 mL × 3). The combined EtOAc extracts were dried over anhydrous Na_2SO_4 and concentrated under a vacuum. The EtOAc extract (21 g) was chromatographed on silica gel 60, 70-230 mesh (400 g), with fractions stepwise eluted with hexanes, ethyl acetate and methanol, yielding Fractions A-G. Bioautography-guided bioassay showed fraction C, D and E exhibited antifungal activities and were selected for further bioassay-guided isolation (Figure 3.16).

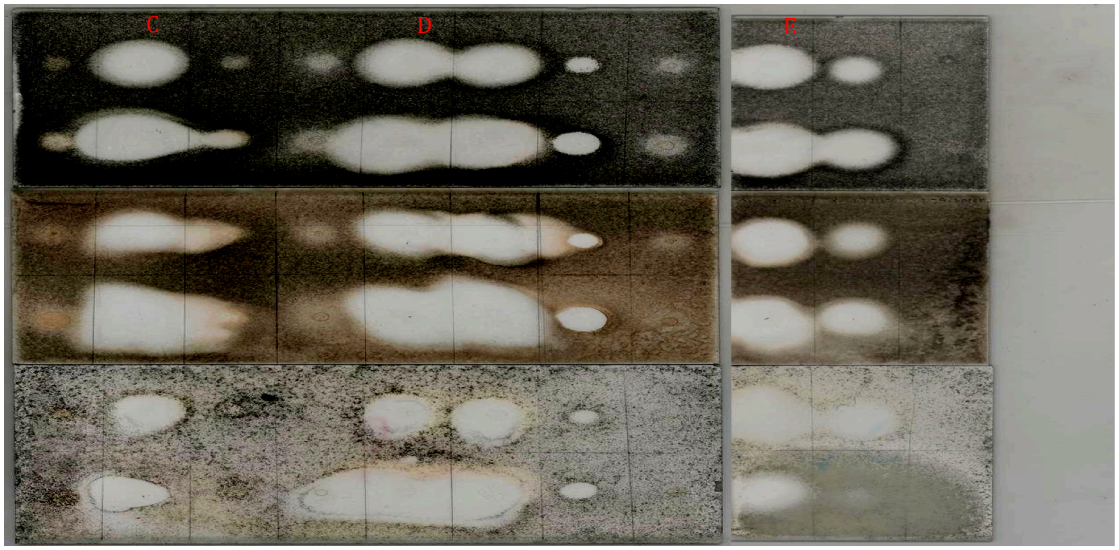
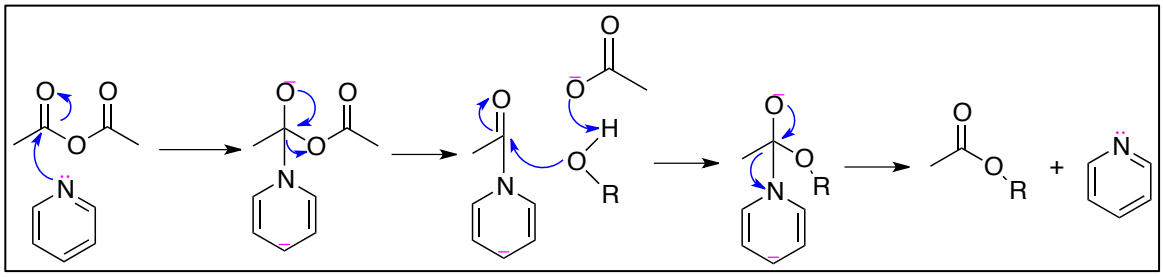


Figure 3.16 Antifungal activities with very clear inhibitory zones of fractions C, D and E from *C. cladosporioides* crude extracts**.**

Fraction C was purified by HPLC on RP-18 using a MeOH-H_2O gradient to afford four compounds, cladosporin (**20**) (5.038 g), isocladosporin (**21**) (8.3 mg), palmitic acid (**29**) (10 mg) and linoleic acid (**30**) (8 mg). Fraction D was separated on Sephadex LH-20 CC eluting with DCM-MeOH (1:1) to afford 128 fractions. Sub-fractions 72-128 were combined to afford 5'-hydroxyasperentin (**26**) (117 mg). Sub-fractions 31-71 were combined and were separated by Biotage Isolera 1-EV system using a $CHCl_3$/MeOH gradient to afford cladosporin-8-methyl ether (**27**) (3 mg) (Figure 3.15). 5'-hydroxyasperentin (50 mg) reacted with 2 mL acetic anhydride and 2 mL pyridine (catalyst) for 24 hours to generate three less polar compounds. These three compounds were purified by preparative TLC (developed with petroleum: EtOAc 1:1) and dried under nitrogen to give one pure compound, 6, 5'-diacetyl cladosporin (**28**, 4.5 mg). The electrons transfer in this reaction was shown as below:



*Pyrindine has a lone pair of electrons on nitrogen which cannot be delocalized around the ring, making nitrogen a common nucleophile and often used in acetylation reactions.

Cladosporin (**20**): white solid; $[\alpha]^{20}_{D}$ = -14.0(c = 1.0, EtOH); mp 175−177 °C; ^{1}H NMR (400 MHz, $CDCl_3$) δ 10.99 (d, *J*= 1.6 Hz, 1H), 8.46 (m, 1H), 6.29 (d, *J*= 2.0 Hz, 1H), 6.16 (d, *J*= 1.6Hz, 1H), 4.71−4.66 (m, 1H), 4.13−4.10 (m, 1H), 3.97 (br s, 1H),2.88−2.75 (m, 2H), 2.02−1.95 (m, 1H), 1.84−1.78 (m, 1H), 1.68− 1.53 (m, 4H), 1.37−1.32 (m, 2H), 1.20 (d, *J* = 6.4 Hz, 3H); ^{13}C NMR(100 MHz, $CDCl_3$) δ 170.1, 164.1, 163.7, 141.6, 106.9, 101.8, 101.0,76.3, 67.8, 66.6, 38.8, 33.4, 30.9, 30.6, 19.0, 18.0; HRESIMS calcdfor $C_{16}H_{21}O_5$ [M + H]+ 293.1384, found 293.1389.

Isocladosporin (**21**): a white solid; $[\alpha]^{20}_{D}$ = −5.0 (c = 1.0, $CHCl_3$); mp 158−159°C; ^{1}H NMR (400 MHz, $CDCl_3$): δ 11.07 (d, *J* = 2.0 Hz, 1H), 7.83(m, 1H), 6.34 (d, *J* = 2.0 Hz, 1H), 6.17 (s, 1H), 4.83−4.78 (m, 1H),3.72−3.66 (m, 1H), 3.49−3.45 (m, 1H), 2.84−2.71 (m, 2H), 1.92−1.71 (m, 3H), 1.60−1.50 (m, 3H), 1.27−1.20 (m, 2H), 1.15 (d, *J* = 6.4Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.3, 164.2, 163.3, 141.9, 106.8, 101.9, 101.3, 76.0, 74.1, 73.2, 41.7, 33.6, 33.1, 31.6, 23.4, 22.0;HRESIMS calcd for $C_{16}H_{21}O_5$ [M + H]+ 293.1384, found 293.1394.

3.3.3.4 X-ray crystallographic study of cladosporin

A single-crystal X-ray diffraction study was conducted on compound **20**. $C_{16}H_{20}O_5$,

MW=292.3, pale yellow needles were obtained with the slow evaporation of a solution in ethyl acetate. A single crystal, approximate dimensions 0.45 × 0.36 × 0.20 mm, was used for data collection on a Bruker Smart Apex II system, using Cu KR radiation with a graphite monochromator, fine-focus sealed tube. The crystal was kept at 100 K under a stream of cooled nitrogen gas from a KRYO-FLEX low-temperature device. Cell dimensions are α = 862.1 (2) Å, b = 6.8656 (3), c = 1427.8 (3), ß = 1168.6 (3) pm, V = 1438.4 (6) 10^6 pm^3. Data collection, indexing, and initial cell refinements were all carried out using APEX II software. All non-hydrogen atoms were visible in the difference electron density maps and refined with riding model at idealized positions. Anisotropic displacement parameters were included in the refinement for all non-hydrogen atoms. Frame integration and final cell refinements were done using SAINT software; 2064 reflexions $4.5 < 2\theta < 52°$, $-2 < h < 10$, $0 < k < 14$, $0 < l < 17$, 2002 independent intensities (Rint = 0.015), no absorption correction. Refinement converged at R1 ($I > 2\sigma(I)$)=0.038, wR2 (all data) = 0.087, S = 1.049, max. (δ/σ) = 0.001, min/max height in final ΔF map -0.16/0.15 e $Å^{-3}$. Structure solution, refinement, graphics, and generation of publication materials were performed using SHELXTL, V6.12 software. Hydrogen atoms were placed in their expected chemical positions using the HFIX command and were included in the final cycles of least-squares refinement, with isotropic U_{ij}'s related to the atoms ridden upon (Figure 3.17).

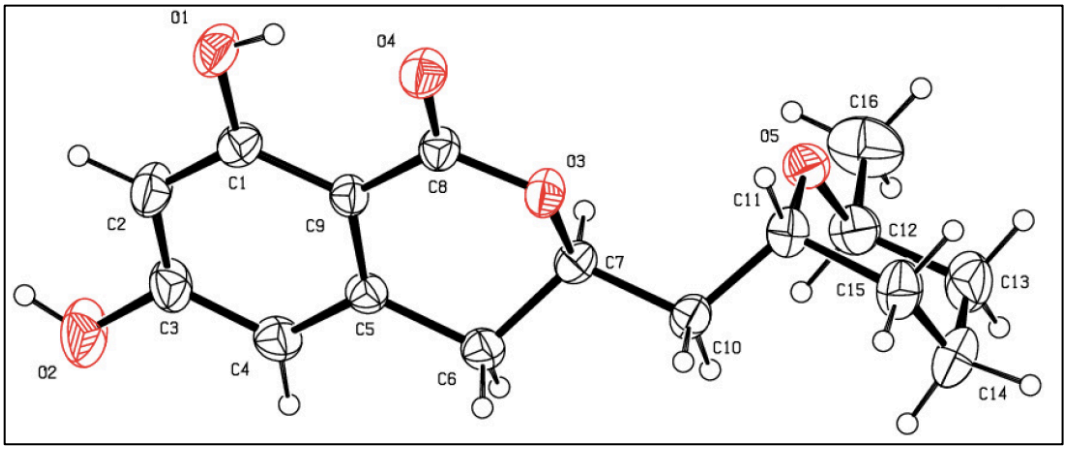


Figure 3.17 Crystal structure of cladosporin (**20**) from *C. cladosporioides*.

3.3.3.5 HPLC spectrum of cladosporin and isocladosporin

A partial of ethyl acetate fraction was purified by HPLC on RP-18 using gradient elution from H_2O/MeOH (starting with 80:20 for 10 min, and then MeOH increased to 100% within 30 min; 100% MeOH for 10 min) with the flow of 8 mL/min. The big two peaks refer to cladosporin and isocladosporin with retention time of 43 min and 44 min, respectively (Figure 3.18).

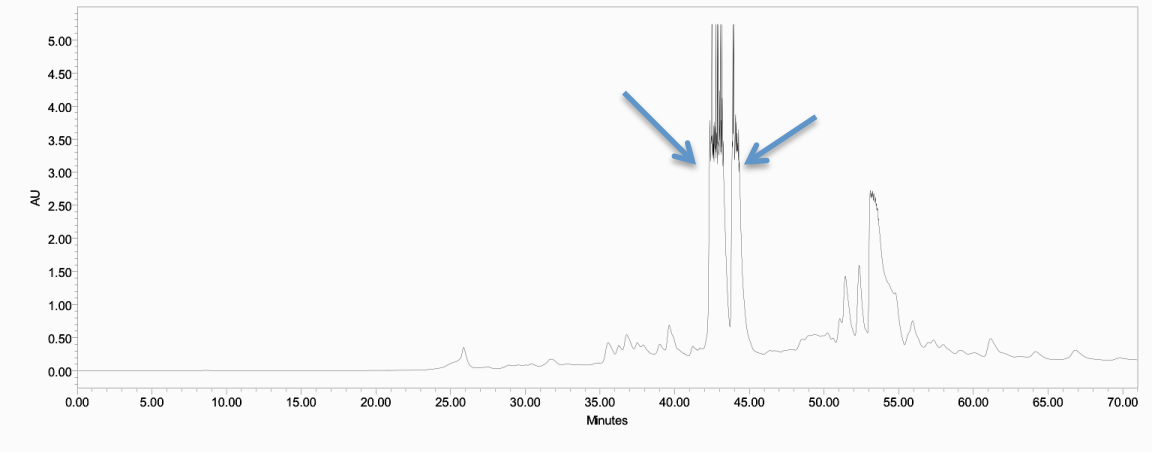


Figure 3.18 HPLC spectrum of cladosporin **(20**, left peak) and isocladosporin **(21**, right peak).

3.3.3.6 Opioid and cannabinoid binding assay

Cell Culture. CHO-K1 cells (ATCC) were stably transfected via electroporation with full length human recombinant cDNA for cannabinoid receptor subtypes 1 and 2 (obtained from Origene). These cells were maintained at 37^{o}C and 5% CO_2 in a Dulbecco's Modified Eagles's medium (DMEM) nutrient mixture F-12 HAM supplemented with 2 mM L-glutamine, 10% fetal bovine serum, 0.5% penicillin–streptomycin, and G418 (600 mg/mL). CHO-K1 cells stably transfected with opioid receptor subtype δ, κ and μ were a generous gift from Roth labs (University of North Carolina at Chapel Hill, Chapel Hill, N.C., U.S.A.). These cells were maintained at 37^{o}C and 5% CO_2 in a DMEM nutrient mixture supplemented with 2mM L-glutamine, 10% fetal bovine serum, 0.5% penicillin–streptomycin, and either G418 (600 mg/mL) or hygromycin B (300 mg/mL). Membranes were prepared by scraping the cells in a 50 mM Tris-HCl buffer, homogenized via sonication and centrifuged for 40 minutes at 13650 rpm at 4^{o}C. These were kept at -80^{o}C until used for binding assays. Protein concentration was found via Bio-Rad Protein Assay [192].

Radio-ligand Binding for Cannabinoid and Opioid Receptor Subtypes. Cannabinoid binding took place under the following conditions: 10 μM of each compound was incubated with 0.6 nM [^{3}H] CP 55,940 and 10 μg CB1 or CB2 membrane for 90 minutes in a salinized 96-well plate. The reaction was terminated via rapid vacuum filtration through GF/C filters presoaked with 0.3% BSA using a Perkin Elmer 96-well Unifilter (Perkin Elmer Life Sciences Inc., Boston, Mass. U.S.A.) followed by 10 washes of 50 mM Tris-HCl. Plates were read using a Perkin Elmer Topcount (Perkin Elmer Life Sciences Inc., Boston, Mass. U.S.A.). Total binding was defined as binding in the presence of 0.1% DMSO. Nonspecific binding was defined as binding observed

in the presence of 10 μM CP55, 940. Specific binding was the difference between total and nonspecific binding. Percent binding was found with the following formula: 100-(Binding of compound- nonspecific binding)*100/Specific Binding. Opioid binding took place under the following conditions: 10 μM of each compound was incubated with [^{3}H]-DAMGO (μ), [^{3}H]-U-69, 593 (κ), or [^{3}H]-Enkephlin (δ) for 60 minutes in a 96-well plate. Tritium and membrane concentration for each cell line was determined by saturation experiments performed after each batch of membrane was scraped. The reaction was terminated via rapid vacuum filtration through GF/B filters presoaked with 0.3% BSA using a Perkin Elmer 96-well Unifilter followed by 10 washes of 50 mM Tris-HCl. Total binding was defined as binding in the presence of 0.1% DMSO. Nonspecific binding was defined as binding observed in the presence of 10 μM DAMGO (μ), nor-Binaltorphimine (κ), or DPDPE (δ). Specific binding was the difference between total and nonspecific binding. Percent binding was found with the following formula: 100-(Binding of compound- nonspecific binding)*100/Specific Binding. Ki and IC_{50} values were calculated using Graph-Pad Prism 5.

3.3.3.7 *In vitro* antimicrobial assay

All organisms used for the biological evaluation of the metabolites from *Eurotium repens* were obtained from the American Type Culture Collection (Manassas, VA) and include the fungi *Candida albicans* ATCC 90028, *Candida glabrata* ATCC 90030, *Candida krusei* ATCC 6258, *Cryptococcus neoformans* ATCC 90113, and *Aspergillus fumigatus* ATCC 204305 and the bacteria *Staphylococcus aureus* ATCC 29213, methicillin-resistant *S. aureus* ATCC 33591 (MRS), *Escherichia coli* ATCC 35218, *Pseudomonas aeruginosa* ATCC 27853, and *Mycobacterium intracellulare* ATCC 23068. Susceptibility testing was performed using a modified version of the CLSI (formerly NCCLS) methods [193]. *M. intracellulare* was tested using

a modified method[194]. Samples were serially diluted in 20% DMSO/saline and transferred in duplicate to 96-well flat bottom microplates. Microbial inocula were prepared by correcting the OD_{630} of microbe suspensions in incubation broth to afford final target inocula. Ciprofloxacin (ICN Biomedicals, Ohio) for bacteria and amphotericin B (ICN Biomedicals, Ohio) for fungi are included as positive controls in each assay. All organisms were read at either 630 nm using the Biotek Powerwave XS plate reader (Bio-Tek Instruments, Vermont) or 544ex/590em, (*M. intracellulare, A. fumigatus*) using the Polarstar Galaxy Plate Reader (BMG LabTechnologies, Germany) prior to and after incubation. Percent growth was plotted versus test concentration to afford the IC_{50}.

3.3.3.8 Herbicidal activity of cladosporin

The lettuce used was Iceberg A Crisphead from Burpee Seeds. Agrostis seeds were Penncross variety (Creeping Bentgrass species) obtained from Turf-Seed, Inc of Hubbard, Oregon. For seed preparation, all seeds were surface sterilized prior to use in any bioassay by mixing with a 5 to 10% Chlorox solution for approximately 10 minutes. Seeds were thorough rinsed with deionized water (Millipore system) and air-dried in a sterile environment. All bioassays were done in duplicate in sterile non-pyrogenic polystyrene 24-well cell culture plates (CoStar 3524, Corning Incorporated). One filter paper disk (Whatman Grade 1, 1.5 cm) was placed in each well to be used. The control wells contained 200 mL of Millipore water. The control + solvent well contained 180 mL of water and 20 mL of the solvent. All sample wells contained 180 mL of water and 20 mL of the appropriate dilution of the sample. Water was always pipetted into the well before the sample or solvent. All plate preparation was done in a sterile environment to lessen chances of any possible contamination. Figure 3.19 showed

cladosporin selectively inhibited the growth monot (agostis) and showed no activity against dicot (lettuce) (Figure 3.19).

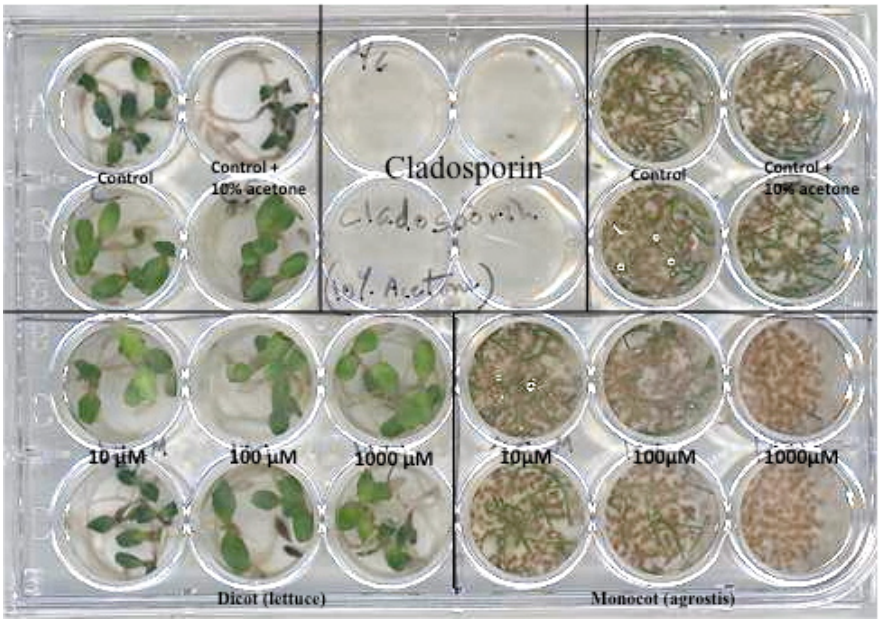


Figure 3.19 Herbicidal activity of cladosporin against monocot (Agrostis)

When prepping lettuce plates, five seeds were placed in each well. Lids were sealed with Parafilm. The plates were incubated in a Percival Scientific CU-36L5 incubator under continuous light conditions at 26°C and 120.1 umol s^{-1} m^{-2} average light intensity. Plates were incubated for at least seven days. Ranking of plant growth was subjective. Ranking was based on a scale of 0 to 5. A ranking of 0 indicated no apparent inhibition (sample well plants looked identical to the Control + Solvent well plants). A ranking of 5 indicated no growth or complete inhibition. A ranking of five was given only if no seeds germinated.

3.4 IDENTIFICATION AND BIOLOGICAL STUDY OF THE FUNGUS MILOGRANTE

The fungus Milograntewas the second fungus chosen for further isolation study. In the bioautography bioassay, it showed very good antifungal activity against three *Colletotrichum* species (Figure 3.8)

3.4.1 Identification of Fungus Milogrante

3.4.1.1 Results

The fungus Milogrante is inoculated in malt extract agar (MEA) after 10 days at 25 ± 2°C, under cool-white fluorescent lights (55 ± 5 μmol/m^2/s) with a 12 h photoperiod, colonies with 23 mm of diameter, expanding, velvety, olivaceous green and reverse olivaceous black (Figure 3.20 a & b). The fungus shown conidiophores 50 µm long, 2-6 µm wide, without swellings, with terminal and lateral ramifications, bearing branched conidial chains, pale olivaceous brown, conidia ellipsoidal to limoni-form, smooth-walled, olivaceous brown, 1-celled, with dark scars (Figure 3.20 c). In addition, the sequence of fungus Milogrante displayed 99% of identity with different sequences of cultivable and non-cultivable fungi deposited in GenBank database. However, according to Gazis*et al.* (2011) [195]and Ko*et al.* (2011) [196], sequencing of the ITS region may fail to recognize some *Ascomycota* taxa and for this reason there are several erroneous fungal sequences deposited in GenBank. To increase the taxonomy accuracy and avoid mistakes on the phylogenetic inferences, the ITS1-5.8S-ITS2 nuclear ribosomal gene sequence of the fungus Milogrante was compared with sequence of type species of *Cladosporium cladosporioides* (Figure 3.20 d). In the phylogenetic analysis, the nucleotides difference among the fungus Milogrante and the sequence of the type species *Cladosporium cladosporioides* CBS 144.35 (HM148012) was of the one nucleotide (0.17 %). Using physiological, morphological

characteristics and molecular techniques, the fungus Milograntewas identified as *Cladosporium cladosporioides* (Fres.) de Vries.

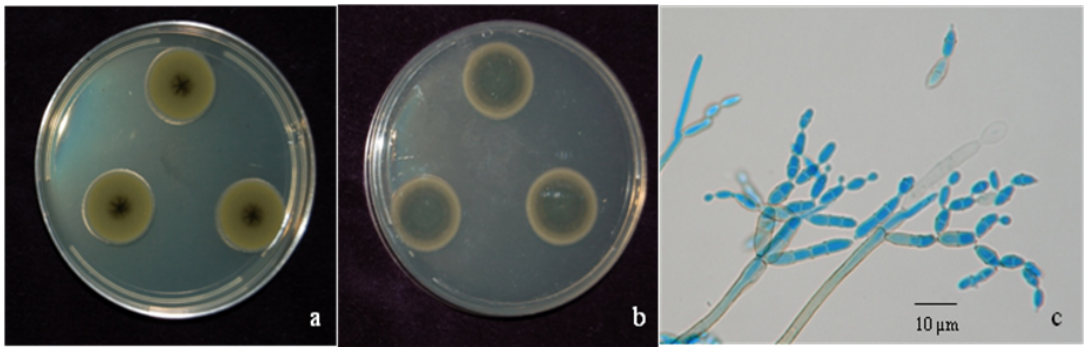


Figure 3.20 Taxonomic data of the fungus Milograntе. (a & b: Morphological verse and reverse aspect of culture after 7 days on MEA at 7 days at 25 ± 2°; c: micromorphological structures (conidiophores and conidia)).

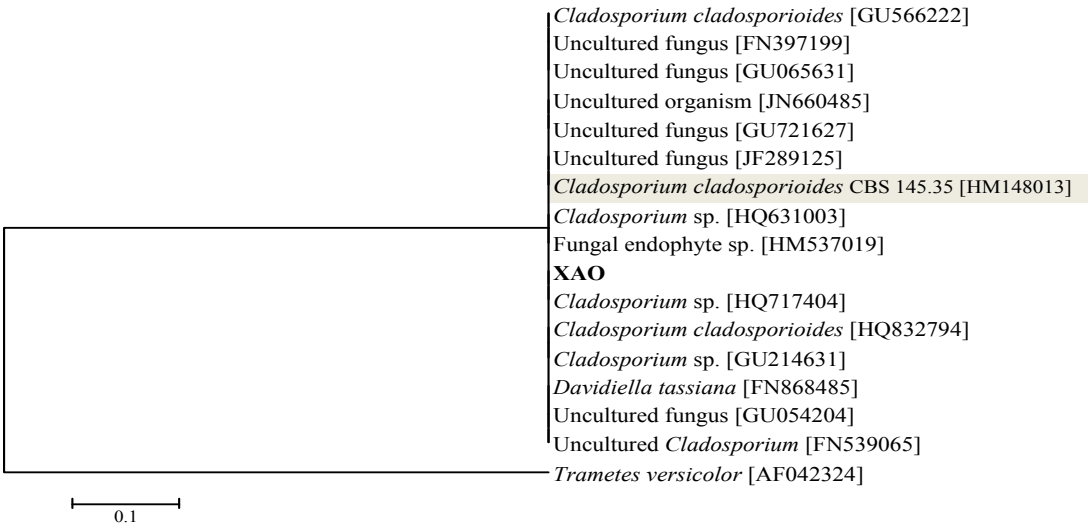


Figure 3.20 d Phylogenetic analysis of the fungus and their nearest relatives. The tree was constructed based on the rRNA gene sequences (ITS1-5.8S-ITS2) by using the maximum composite likelihood model; the tree was rooted with *Trametes versicolor* [AF042324] as an outgroup. [T]sequence of type species is shown in gray box.

3.4.1.2 Phylogenetic analysis

Genomic DNA from the fungus Milogranteas extracted with DNeasy Plant Mini Kit (Qiagen Inc., Valencia, CA) and used as a template in PCR amplifications. The ITS1-5.8S-ITS2 genomic region (ITS) was amplified from genomic DNA using the forward primer ITS1 (5´-TCCGTAGGTGAACCTGCGG-3´) and the reverse primer ITS4 (5´-TCCTCCGCTTATTGATATGC-3´) [197]. PCR amplifications were carried out in 50 µL reaction mixture containing 1x PCR reaction buffer, 0.2 mM dNTP mixture, 0.2 µM of each forward and reverse primers, 1.5 mM $MgSO_4$ and 2 U of Platinum Taq DNA Polymerase (Invitrogen, CA). The PCR program consisted of one initial denaturation step at 94°C for 3 min followed by 40 cycles at 94°C for 30 sec, 50°C for 30 sec, 72°C for 1:30 min, with a final extension at 72°C for 7 min. PCR was performed in an M&J Research Gradient Cycler PTC-225. After amplification, an aliquot was analyzed by electrophoresis on a 1% TAE agarose gel, visualized under UV light and PCR products were compared to the molecular size standard 1kb plus DNA ladder (Invitrogen, city CA). Successfully amplified PCR products were extracted using MinElute PCR Purification Kit (Invitrogen, city CA) and sequenced on an automated DNA Sequencer (model ABI 3730XL; Applied Biosystems, Foster City, CA). Consensus sequence data of the endophytic fungus was submitted to the GenBank database under the number NRRL 5507.The sequence obtained was submitted to phylogenetic inferences, which were estimated using MEGA Version 5.0 [198]. The maximum composite likelihood model was used to estimate evolutionary distance with bootstrap values calculated from 1,000 replicate runs. The sequence of the type fungal species and some references sequences deposited in GenBank were added to phylogenetic analysis accuracy. Information about the fungal taxonomic hierarchical levels follows the databases MycoBank (www.mycobank.org) and Index Fungorum (www.indexfungorum.org).

3.4.1.3 Physiological and morphological analysis

The physiological and morphological characteristics of the fungus Millogrante were observed on 2% malt extract agar ((malt extract 2%, peptone 0.1%, glucose 1.5% and agar 2% (MEA)). The colony diameter was measured on media, which was inoculated in three-point cultures and incubated for 7 days at 25 ± 2°C under cool-white fluorescent lights (55 ± 5 μmol/m^2/s) with a 12 h photoperiod to sporulation. A duplicate set of MEA plates was incubated in the dark at 25°C to microscopic parameters determination using slide cultures mounted in methyl blue in polyvinyl-lactophenol.

3.4.2 Biological Study of Isolate 2 of *C. cladosporioides* (Milogrante)

NMR-guided isolation showed that almost all the active constituents in isolate 2 are fatty acids, which is totally different from that in isolate 1. Fatty acid refers to a carboxylic acid bearing a long unbranched aliphatic chain. The long chain can be monounsaturated, polyunsaturated, or saturated with even number of carbons in the majority of naturally occurring fatty acids. Fatty acids play important roles in our daily life. As an important part of a healthy diet, they keep skin healthy, prevent early aging and promote weight loss [199]. They also aid cell membrane development, strength, and function, which is essential for organs and tissue. Some polyunsaturated fatty acids are known to be major components of the human central nervous system (CNS). For example, Docosahexaenoic acid (DHA) and arachidonic acid (AA) are major components of the CNS, where they play a critical role in neural development. DHA has been shown to be essential in the prevention of numerous diseases. Low levels of DHA may lead to Alzheimer's disease. Arachidonic acid (AA) is another important fatty acid, which is a key inflammatory intermediate [200, 201, 202].

In recent years, more and more natural lipids have been identified as endogenous leads for the central cannabinoid receptors which belong to family of GPCRs and are associated with many processes including pain, craving, neurodegeneration, metabolic regulation, anxiety and immune function [203, 204]. So discoveries in cannabinoid receptors have provided an exciting future for cannabinoid receptor-based therapies to treat a variety of disorders in CNS [205]. Linoleic acid is an omega-6 essential polyunsaturated fatty acid and its deficiency can result in symptoms such as hair fall, dry hair, and poor wound healing [206]. Palmitic acid is one of the most common saturated fatty acids found in animals and plants and mainly used to produce soaps, cosmetics, and release agents [207].

3.4.2.1 Opioid and cannabinoid receptor binding affinity

The EtOAc crude extract (36 g) of the isolate of *C. cladosporioides* (Milogrante) was chromatographed on silica gel 60, 70-230 mesh (400 g), with fractions stepwise eluted with hexanes, ethyl acetate and methanol, yielding seven fractions (fractions 1-7). The crude extracts and their fractions were submitted for opioid and cannabinoid receptor testing. The result showed that fractions 2 (Hex: EtOAc 1:1) showed promising activities against all three opioid receptors (δ, 92.80; μ, 95.07; and κ, 91.98) and fraction 3 (100% EtOAc) and fraction 6 (100% MeOH) showed modest activities against δ receptor. The crude extracts and fractions 1 (100% Hexanes), fraction 4 (MeOH: EtOAc 25:75), fraction 5 (MeOH: EtOAc 50:50) and fraction 7 (MeOH: H_2O 50:50) showed weak or no binding affinities for opioid receptors. All the fractions had no CB1/CB2 affinities. And then, fraction 3 (3.14 g) was chromatographed on liquid silica column to give 14 sub-fractions which were subsequently tested by opioid and cannabinoid receptor assays. The result showed 3 sub-fractions (DCM/EtOAc 60/40 (δ, 78.4); DCM/EtOAc50/50 (δ, 70.84); DCM/EtOAc40/60 (κ, 59.82)) had affinity for δ or κ receptor (Table 3.4).

Table 3.4 Binding Affinity Assay of the Fungus Migrante for Human Opioid Receptors and Cannabinoid receptors.

Species	Fractions (10 µg/ml)	Opioid Receptors (%)			Cannabinoid Receptors (%)	
		δ	κ	µ	CB1	CB2
XW-Migrante	Crude extracts	20.38	49.06	44.23	43.2	8.7
	100% Hexanes	-2.71	23.15	25.14	-24.20	-38.24
	Hex:EtOAc (1:1)	92.80	91.98	95.07	-70.63	-25.87
	EtOAc	53.96	20.27	38.47	-52.83	-42.32
	MeOH:EtOAc (25:75)	47.90	6.58	22.14	-59.96	-17.36
	MeOH:EtOAc (1:1)	33.85	4.41	35.75	-55.80	-16.95
	100% MeOH	57.16	7.30	47.45	-67.04	1.60
	MeOH: H_2O	39.67	13.78	33.30	-44.11	6.71
	DCM:EtOAc (6:4)	78.4	-	-	-	-
	DCM: EtOAc (1:1)	70.84	-	-	-	-
	DCM:EtOAc (4:6)	-	59.82	-	-	-
Naloxone (10 µm)		89.15	98.65	99.42	-	-
CP 55,940 (10 µm)		-	-	-	105.75	100.19

3.4.2.2 Antifungal study

The crude extracts, fractions and sub-fractions from the isolate 2 of *C. Cladosporioides* were tested for their antifungal activity against three *Colletotrichum* species using bioautography. The crude extracts and fractions 6 (100% MeOH) and fraction 7 (MeOH: H_2O 50:50) showed very good inhibition zones against *C. fragariae* (Figure 3.22).

Figure 3.22 Bioautographic result of fraction 6 and fraction 7 against *C. fragariae*. Four µL of 20 mg/mL with 1 cm band size of fraction 6 and fraction 7 were applied at 1 cm measured from the bottom edge of the TLC (10 × 10 cm) and developed with $CHCl_3$: MeOH (7:3).

Fractions 6 were purified using gradient HPLC with acetonitrile and water. Two fractions were obtained at 20.93 min and 21.77 min (Figure 3.23). They were tested to exhibit promising antifungal activity against *C. fragariae* (Figure 3.24). NMR data showed that they all belonged to fatty acids.

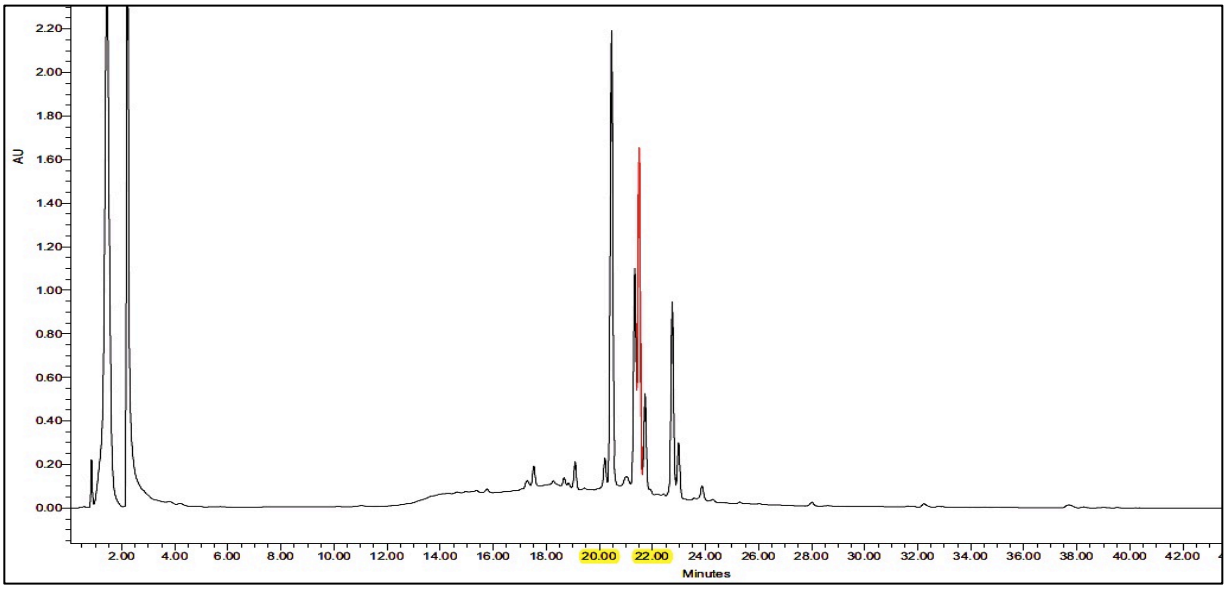


Figure 3.23 HPLC spectrum of two fatty acids (20.93 min, 21.77 min) from fraction 6**.**

Figure 3.24 Bioautographyic result of two fractions from fraction 6**.**

After GC/MS analysis, these two fractions were determined as linoleic acid and palmitic acid. The mixtures of these two acids with different ratios were tested for the binding affinity for human opioid receptors (μ, δ, κ) (done by Dr. Mohamed Radwan and Amer Tarawneh). The

result showed that the mixture of linoleic acid and palmitic acid with 1:1 ratio was the most potent with an IC_{50} value of 7.65 μM. This led us to believe that active components of these two acids are working synergistically to produce their effects. To our best knowledge, this is the first time to report opioid binding activity of these two acids. Besides the great potency of linoleic and palmitic acids, they offer greater safety in their application than the synthesized or semi-synthesiezed drugs. So these lipids need to be further exploited.

CHAPTER 4 DEVELOPMENT OF A MINATURISED 24-WELL STRAWBERRY LEAF DISK BIOASSAY FOREVALUATION OF NATURAL FUNGICIDES

4.1 INTRODUCTION

Effective disease control strategies for crop plant pathogens rely on the use of fungicides. Wedge *et al*. reported on new fungicide management strategies for control of strawberry fruit rot diseases in the Gulf state region and the need for efficacious fungicides to provide growers with more options for controlling diseases [208]. Disadvantages of the use of these chemical controls include the development of resistant fungal strains as well as potential environmental and mammalian toxicities. Resistance of many plant pathogens to commonly used commercial fungicides is becoming a serious problem and has limited the number of effective disease control agents. For example, numerous isolates of *Colletotrichum* spp. developed benomyl insensitivity (only showed 40%-60% fungus growth inhibition) after several years of use to protect strawberry and other fruit crops [209]. *Colletotrichum* species often cause typical symptoms of anthracnose, a disease characterized by sunken necrotic lesions usually bounded by a red margin [210]. As we mentioned before, anthracnose diseases of strawberry (*Fragaria* × *ananassa* Duch) are serious problems for fruit and plant production in many areas of the world [211]. The pathogens, *Colletotrichum fragariae* A. N. Brooks*, C. gloeosporioides* (Penz.) Penz*.,* and *C. fragariae* CF - 75, can occur singly or in combination to infect flowers, fruit, leaves, petioles, stolons, and crowns [212].

In the continued fungicide discovery, we are focused on natural products derived from plants and fungal organisms with emphasis on compounds with fungicidal activity[213]. Natural antifungal agents are generally broad-spectrum compounds with low mammalian and environmental toxicity and 3-dimensional structures that synthetic compounds may lack. Along with discovery of new molecules with novel motion of action and our interest in antimicrobial agents from higher plants and fungi [17], a novel miniaturized bioassay for evaluating small amounts of available compounds is also needed [214].

Development of new plant protectants with a lipophilic nature is limited by the lack of suitable bioassays to properly evaluate these compounds. While the lipophilic nature of phytochemicals allows them to embed themselves into the leaf waxes and persist through rainfall, hence making them potentially useful agrochemicals, this characteristic also makes them very difficult to evaluate in many aqueous bioassays. Examples include essential oils and many marine extracts, which are hydrophobic and insoluble in most aqueous-based antimicrobial disc diffusion and micro-dilution broth bioassays. Direct bioautography on silica gel is our preferred primary screening bioassay and often the best assay for evaluating lipophilic compounds as fungicides for agricultural use, because this assay more closely mimics a leaf surface. Classical leaf bioassays, used to test fungicide efficacy at concentrations between 625 and 2500 ppm, may use 75-100 mg of an experimental compound that is often difficult to obtain. Therefore, the need for a novel miniaturized leaf disk bioassay that utilizes small sample sizes, has high throughput capability, and replicates the real leaf surface is of utmost importance to natural product fungicide discovery. This study presents information about developing of a new 24-well detached leaf bioassay for evaluation of natural fungicides.

4.2 RESULTS AND DISCUSSION

4.2.1 Salt Solutions For 24-well Leaf Disk Assay

Leaf color index of 2.67 obtained after inspecting 7-day-old leaf disks indicated that half strength Hoaglands No. 2 basal salt media (*Phyto*Technology Laboratories) provided excised leaf discs with the best physiological support (Table 4.1). Murashige and Skoog basal salts (Sigma-Aldrich) demonstrated the lowest color index of 1.83. While 2-N-morpholino ethane sulfonic acid (MES, Sigma-Aldrich) and water (control) had a color index of 2.42 and 2.83 respectively. However, MES and water did not gel sufficiently to provide adequate physical support to keep leaf disks afloat for subsequent inoculation with the conidial solutions. Typical working concentrations for Phytagel™ are 1.5-2.5 g/L in plant tissue culture media and up to 10 g/L in microbiological media. Phytagel™ requires the presence of cations (especially divalent) for gelling to occur. Concentrations of calcium and magnesium contained in most plant tissue culture media are typically sufficient for gelation. Low-salt media formulations, especially those used in microbiological applications may require supplementation with additional calcium or magnesium salts (e.g., $CaCl_2$ or $MgSO_4$) or higher concentrations of Phytagel. Therefore we chose ½ strength Hoaglands No. 2 basal salt media containing 1% Phytogel as the best medium to conduct all subsequent 24-well leaf disk studies.

Table 4.1 Half strength Hoagland's No. 2, 2-N-morpholino Ethanesulfonic Acid (MES), Murashige and Skoog Basal Salt Mixture (M&S) and Water (control) Were Evaluated for Their Ability to Sustain Excised Leaf Discs for 7-10 days.

Salt Solution	Leaf Color Value (1-3)	Standard Error
H_2O	2.83 a	0.11
½ Hoagland's	2.67 a	0.22
M&S	1.83 ab	0.30
MES	2.42 b	0.19

Mean leaf color index on a scale from 1-3 (1 = light green, 2 = medium green, 3 = dark green) was used to describe leaf chlorophyll content. Means followed by the same letter are not significant at $p<0.05$.

4.2.2 Pathogen Type, Surfactant, and Inoculum Volume

Colletotrichum fragariae (CF75), *C. fragariae* (CF63), and *C. gloeosporioides* (CG162) were each evaluated for their ability to produce disease lesions under *in vitro* conditions at three levels of Tween 20 (0.3 %, 0.5 %, and 0.7%). Mean necrosis indicated that *Colletotrichum fragariae* (CF75) and *C. gloeosporioides* (CG162) produced the highest number of lesions under *in vitro* conditions and *C. fragariae* (CF63) produced the lowest number of lesions (Table 4.2). The best treatment combination was *C. fragariae* (CF75) where inoculum amended with 0.5% and 0.7% Tween 20 produced 16.72 and 15.53 % necrosis of the leaf disks respectively. *C. gloeosporioides* (CG162) showed no significant differences in leaf lesions between the three Tween 20 concentrations. Lesion development (necrosis) indicated that there was no difference

between 10 or 20 µL inoculum volumes for any of the three *Colletotrichum* spore suspensions when applied to the leaf disk disks.

Table 4.2 Percent mean necrosis was used to evaluate the ability of *Colletotrichum fragariae* (CF75), *C. fragariae* (CF63), and *C. gloeosporioides* (CG162) and three concentrations of Tween 20 (0.3 %, 0.5 %, and 0.7%) in the inoculum solution to produce disease lesions on 15 mm leaf disks.

Fungal isolate	&% Tween 20	% Mean Necrosis	Standard Error
CF63	0.3	7.34 cde	2.53
CF63	0.5	4.84 efg	1.12
CF63	0.7	5.78 def	2.05
CF75	0.3	10.78 bcd	2.13
CF75	0.5	16.72 a	0.93
CF75	0.7	15.53 ab	8.52
Cg162	0.3	12.47 abc	3.27
Cg162	0.5	11.58 abc	2.02
Cg162	0.7	12.00 abc	3.20
H_2O	0.3	0.56 fg	0.00
H_2O	0.5	0.14 g	0.00
H_2O	0.7	0.00 g	0.00

Means followed by the same letter are not significant at $p<0.05$.

4.2.3 Phytotoxicity

Phytotoxicity evaluations were made visually at 12 hrs after treatment of the fungicidal solution or solvent control and prior to inoculation (**Figure 1**). *Origanum onites* essential oil above 1250 ppm demonstrated a 4+ phytotoxicity ranking and *A. sinensis* essential oil demonstrated 1+ phytoxicity ranking at 2500 ppm. Percent healthy tissue, percent leaf necrosis and percent phytotoxicity of the leaf disk are presented in Table 4.3. The commercial fungicide azoxystrobin at 625 ppm had the greatest level of disease control efficacy at 99% healthy tissue. Even at 2500 ppm, azoxystrobin showed the lowest level of phytotoxicity (0.45%). Both *O. onites*and and *A. sinensis* essential oils were phytotoxic at higher doses (Figure 4.1 and 4.2). Color analysis of Plate 1b (Figure 4.2) showed healthy area in green, diseased area in black and phytotoxicity in gray. *Origanum onites* essential oil was more phytotoxic to strawberry leaves than was *A. sinensis* essential oil. *Angelica sinensis* essential oil was a more effective antifungal agent against all three *Colletotrichum* species.

Table 4.3 Percent Healthy Tissue, % Leaf Necrosis, and % Phytotoxicity as Determined at Five Days After Inoculation by a Lemnatec Lemna HTS and SAW Scanalyzer Analyzing Software.

	Azoxystrobin			Essential oil of Origaniumonites			Essential oil of Angelica sinensis			
	625 ppm	1250 ppm	2500 ppm	625 ppm	1250 ppm	2500 ppm	625 ppm	1250 ppm	2500 ppm	Control
% Healthy Tissue										
CF75	99.29	98.93	99.16	65.03	9.79	16.43	92.59	79.65	36.73	86.18
CF63	99.55	99.51	99.64	96.17	7.32	20.94	96.02	72.20	16.18	92.22
Cg162	99.65	99.21	99.80	98.66	33.72	17.42	98.53	97.33	27.64	94.27
% Leaf Necrosis										
CF75	0.40	0.55	0.39	25.81	29.78	1.02	6.49	8.78	9.59	13.01
CF63	0.13	0.23	0.03	3.39	17.17	0.33	2.89	7.58	0.09	6.85
Cg162	0.04	0.40	0.09	1.13	15.97	7.19	1.05	1.42	0.91	4.85
% Phytotoxicity										
CF75	0.31	0.52	0.45	9.16	60.44	82.55	0.92	11.57	53.67	0.80
CF63	0.31	0.26	0.33	0.44	75.51	78.72	1.09	20.21	83.73	0.93
Cg162	0.31	0.39	0.11	0.21	50.31	75.39	0.42	1.25	71.44	0.88

Plate 1

Plate 2

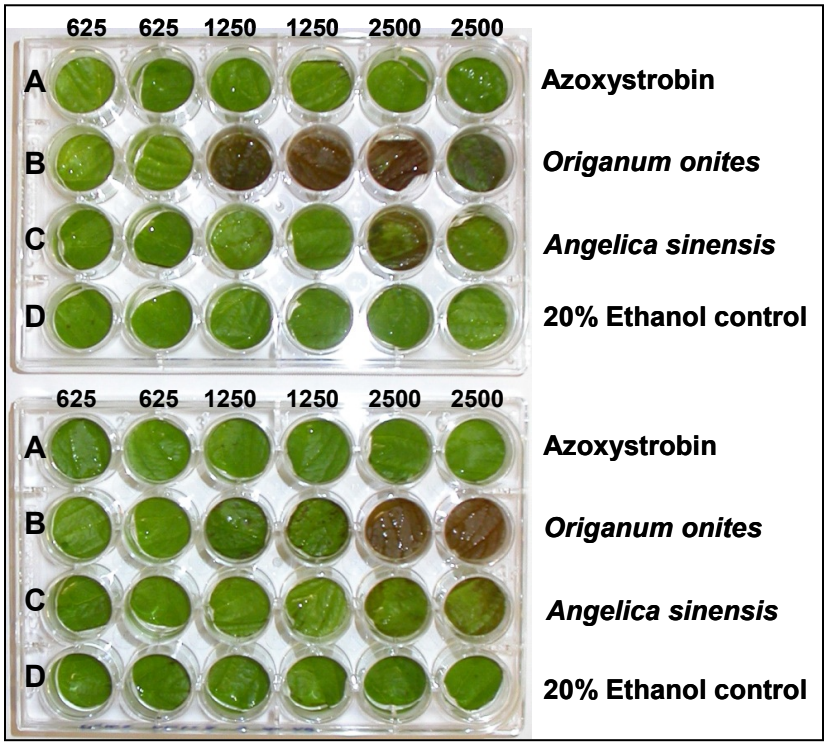


Figure 4.1 Phytotoxicity produced by *Organium onites* and *Angelica sinensis*. 15-mm excised leaf disks from anthracnose susceptible strawberry cultivar 'Chandler' demonstrate easy to visualize 4+ phytotoxicity of *Organium onites* essential oil above 1250 ppm. *Angelica sinensis* essential oil demonstrated 1+ phytoxicity at 2500 ppm. Plate 1 and plates 2 are duplicate treatments showing subtle variation in phytotoxicity bleaching between replicate plates.

Plate 1a

Plate 1b

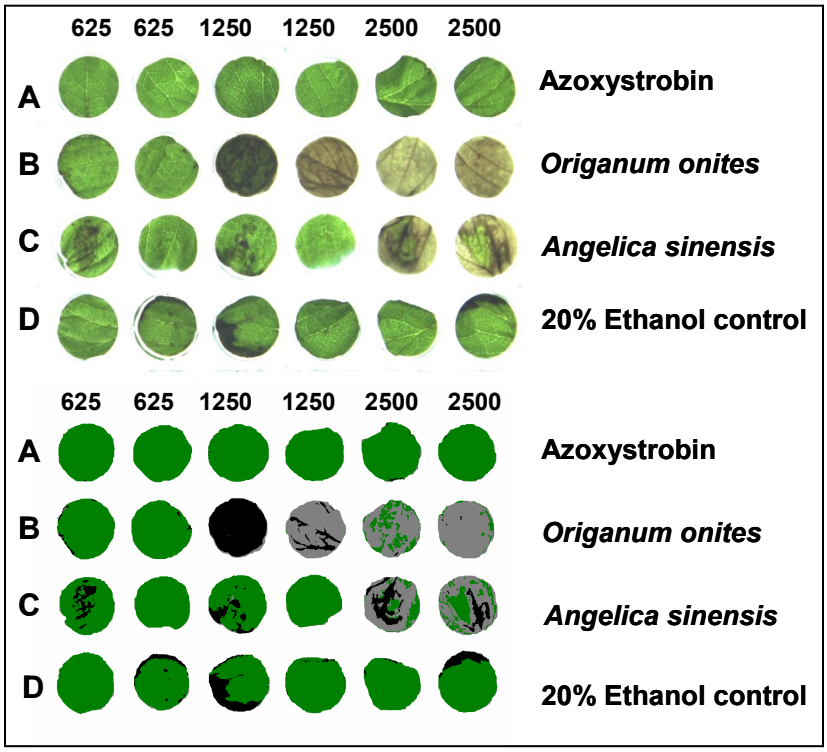


Figure 4.2 Photographic image of the phytotoxicity produced by *Organium onites* and *Angelica sinensis.* Plate 1a is a photographic image of 15-mm excised leaf disks from anthracnose susceptible strawberry cultivar 'Chandler' following 3-5 second dip in fungicide test solutions in a dose-response format and inoculated 12 hrs later with CF75**.** Plate 1b is an image analysis of Plate 1a using the LemnaTec and SAW Scanalyzer analyzing software showing healthy tissue in green, diseased (necrotic) lesions in black, and bleached tissues (phytotoxicity) in gray.

4.3 EXPERIMENTAL

4.3.1 Plant Preparations

Plants of the anthracnose-susceptible cultivar Chandler were obtained in October 2007 from Shingleton Farms (Statonsburg, NC, USA), planted into 14 cm plastic azalea pots containing Metromix 350 (BWI- Memphis, TN), and placed in the greenhouse without shade. Plants were watered daily and Peter's 20-20-20 nutritional solution (BWI- Memphis, TN) was applied weekly. Plants with any signs of disease were rouged and removed from the greenhouse daily.

4.3.2 Leaf Disc Preparation

The first or second fully expanded leaves were removed from stock strawberry plants and transported directly to the laboratory so that the leaves were available for the bioassay in less than 4 hours. Immediately after collection, the leaves were placed in a tray lined with moist paper towels and the tray was closed to retain near 100% relative humidity (RH) and maintained at ~12°C. Only leaves with no visible signs of injury or symptoms of disease were collected. Whole leaves were disinfested by placement in a beaker with 2.5% Clorox for 3 min., then they were rinsed 2-3 minutes in sterile distilled de-ionized water three times. Leaves with any signs of bleaching were discarded. Strawberry leaf disks were then cut from the disinfested leaves using a 15-mm stainless steal cork borer and placed in moisture plate until they were placed in the 24-well plate.

4.3.3 24-well Leaf Disk Assay

Three commercial in vitro salt solutions were evaluated for their ability to maintain leaf disks during the in vitro experiment: half strength Hoagland's No. 2 (H, Phytotechnology Laboratories, Shawnee Mission, KS), 2-N-morpholino ethanesulfonic acid (MES, Sigma M8250, Sigma-Aldrich, St. Louis, MO), Murashige and Skoog basal salt mixture (MS, Sigma M5524). Water (control) was also evaluated for its ability to sustain excised leaf discs for 7-10 days. Stock solutions containing 1% Phytogel (Sigma P8169) were prepared as follows: Hoagland's No. 2 solution (0.815 g/L), MES (195.2 mg/L) containing 5 mL Gamborg's Vitamins (Thiamine-HCl 5mg/L, Pyridoxine-HCl 0.5 mg/L, Nicotinic acid 0.5 mg/L, Myo-inositol 50mg/L), and MS (2.2 g/L). All support solutions were adjusted to 6.5 pH using 0.1 N and 1.0 N NaOH. Excised leaf disks were placed onto the 1500 μL of either respective solidified salts/medium contained in each well of the 24-well plate. Plates were maintained in the sterile biological hood over night prior to pathogen inoculation to allow time for the leaf disks to initiate wound healing and acclimate to the *in vitro* system. The plates were sealed with paper-based tape and placed in the incubator under (55±5 $\mu mols \cdot m^{-2} \cdot sec^{-1}$) (UFL-F17T8/741, Interlectric Corp., Warren, PA) with a 12 h photoperiod in a growth chamber at 25º C for one week. All samples were duplicated and the experiments were repeated three times.

4.3.4 Pathogen and Inoculum Preparation

Three *Colletotrichum* isolates were evaluated for their ability to produce disease lesions under the *in vitro* conditions of this study. *Colletotrichum fragariae* (CF75), *C. fragariae* (CF63), and *C. gloeosporioides* (CG162) isolated from strawberry were evaluated for their ability to produce anthracnose symptoms on excised strawberry leaf discs. Each *Colletotrichum* isolates was grown on 1/2 strength potato dextrose agar (PDA) in 9-cm Petri dishes and incubated under

Ushio Ultra 8 fluorescent lights (55±5 μmols·m^{-2}·sec^{-1}) at 25 °C as previously described (Abril et al., 2008). Conidia were harvested from 7- to 10-day-old cultures, and the aqueous conidial suspensions were filtered through sterile Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) to remove hyphae. Conidial concentrations were determined photometrically from a standard curve based on absorbance at 625 nm [215] and then stock suspensions were adjusted with sterile water to a concentration of 10^6 conidia / mL. Conidial suspensions for inoculation were then adjusted to a final concentration of 10^5 conidia/mL. Two inoculum volumes were evaluated where the inoculum volume applied to each leaf disk was either 10 or 20 μL of any of the three fungal spore suspensions. After leaf disk inoculation, the 24-well plate was sealed with paper-based tape again and placed in the incubator for 6 days. Three concentrations of Tween 20 were also evaluated for their effects on promoting pathogen infection of the leaf disk. Tween 20 concentrations of 0.3%, 0.5%, and 0.7% were prepared in 100 mL sterile distilled water.

4.3.5 Disease Assessment and Phytotoxicity

Experimental compounds were evaluated in a dose-response format. Azoxystrobin that has protective and some curative activity were used as standards for comparison. A 20% ethanol: water solvent control is used in each study to solubilize lipophilic extracts. *Angelica sinensis* roots were purchased as a plant material from Jincheng Lingyi Drugstore, Weinan City, Shaanxi Province, China. *Origanim onites* essential oil was purchased from ALTES Company, Turkey. Stock solutions containing 625, 1250, and 2500 ppm were prepared for azoxystrobin, *Origanum onites* essential oil, *Angelica sinensis* essential oil and 20% ethanol solvent control in 50 mL capped test tubes.

Strawberry leaf disks prepared as previously described were dipped in antifungal test solutions for 3-5 seconds, excess solution was allowed to drain onto the edge of the test tube and

then placed into the appropriate row and column of the 24-well plates. Plates were incubated over night at room temperature and inoculated the next morning with either 20 uL of conidial solution obtained from either *C. fragariae* (CF75), *C. fragariae* (CF63), or *C. gloeosporioides* (CG162). Percent healthy tissue, percent leaf necrosis, and percent phytotoxicity were determined at 5 days after inoculation by a LemnatecLemna HTS and SAW Scanalyzer analyzing software (LemnaTec GmbH, Wurselen, Germany). The instrument was set up to photograph (Sony DFW-SX900 camera) a 24-well plate containing the leaf disks using both top lighting and bottom lighting (8 W fluorescent tubes) and optimized for disk clarity and color. The software configurations were set to measure the area of each disk separately and classify the colors detected within each leaf. Color classification was set to differentiate healthy tissue, bleached tissue, and necrotic lesions on the leaf surface.

SAS (Statistical Analysis System, Cary, N.C.) was used to conduct analysis of variance and mean separation tests on all three experiments. A completely randomized design was used for all experiments. All factors were considered to be fixed effects, except for surfactant concentration in the surfactant experiment. Fisher's protected LSD was used to separate means [216].

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LIST OF APPENDICES

Appendix: A

Protocol 1. Preparation of ½ Strength Potato Dextrose Agar (PDA)

Weigh/measure (using weighting boat on electronic balance 1/10000 gram)

1. 19.6 g PDA Powder (Difco-Becton, Dickinson and Company)
2. 8 g Bacto Agar (Difco-Becton, Dickinson and Company)
3. 1000 mL DDI H_2O (Ultra Pore Millipore System)
4. Add to Flask, Heat, and Stir
5. Stirs suspension until completely dissolve--Divide into 2 Erlenmeyer flasks (500 mL)--Cover the flask with aluminum foil & Autoclave tape – Autoclave at 121 ^{o}C for 45 min

Protocol 2. Preparation of ½ Strength Potato Dextrose Agar/Oatmeal (PO)

1. 24 g PDA: OMA mixture
2. 8 g Bacto Agar
3. 1000 mL DDI H_2O
4. Erlenmeyer flask /magnetic stirring bar on Heat/stirrer
5. Stirs suspension until completely dissolve--Move 1000 mL into 500 mL Erlenmeyer flasks cover the flask with aluminum foil.
6. Autoclave at 121 ^{o}C for 45 min (don't forget the autoclave tape).

Protocol 3. Preparation of Potato Dextrose Broth (PDB)

1. 12.5g PDB Powder
2. 0.5g Bacto Agar
3. 500mL DDI H_2O (Ultra Pore Millipore System)
4. 0.5mL Tween 80 (add it after upper materials are dissolved)
5. Place on a stir plate and heat. While stirring and heating, pipette 0.5 mL of Tween 80 into flask with magnetic stirring bar.
6. Stirs suspension until completely dissolve--Move 100mL into 250mL Erlenmeyer flasks cover the flask with aluminum foil – Autoclave at 121 ^{o}C for 45 min (don't forget the autoclave tape).
7. Place 100 mL aliquots in the refrigerator.
8. Filter before use of spray (0.45 microns)

Protocol 4. Streaking Plates

1. Remove media plates from the refrigerator to be inoculated.
2. Allow plates to warm to room temperature.
3. Condensation on the plate lids, place the plates on the back of the hood with the lid slightly opened to allow the condensation to evaporate.
4. Once there is no more condensation on the lid, close the plates.
5. Label the bottom plate of the petri dish.

   i.e. ½ PDA *C. fragariae* fr. Stock 7-12-12 6-03-12 (today's date)
6. Add 50 µL of the inoculums to each plate.
7. Streak the plate with a sterile inoculun loop. Change lops between each plate that is being

streaked to reduce contamination.

8. Wrap the inoculated plate with 2 pieces of parafilm.
9. Place the inoculated plate in the appropriate growth chamber with the lid side facing up.

Protocol 5. Procedures of Fungus Harvest

1. Remove fungal isolates inoculated 7-10 days from Percival Growth Chamber @ 24 °C (incubation period).
2. Test the contaminated situation of the fungi, discard the green ones, reserve the black ones and discard the black parts.
3. Label sterile test tubes and the cuvettes according to fungal isolates before harvesting (using the corresponding color codes).
4. Use different color label bands label each different fungal isolate to avoid mistakes.
5. Place sterile funnel into glass test tube using forceps.
6. Remove miracloth square from sterile wrap, pouch and place into funnel.
7. Place a fungal isolate (*e.g.* Ca Goff) plate onto turn table -----pipette 10 ml of sterile DDI H_2O into the plate ----using L cell spreader softly remove conidia from media.
8. Using Gilson 10ml Pipetman, pipette conidia suspension through Miracloth, seal test tube with Parafilm square, and then place tube with suspension in ice bucket.
9. Prepare label microscope slide (size 25×75×1mm) and check for contamination using Olympus BX60 (pipette 15 μL of conidia suspension onto slide /cover by using cover glass).
10. Examine: prepare conidia suspension.
11. Collect spore suspension with pipette (10 mL) from media into sterile tube and remove test tubes from ice bucket and place in refrigerator until over night for harvesting spore

deposition.

Protocol 6. Preparation of RPMI buffer for Stock Solution.

To prepare 1 liter liquid medium:

1. Measure out 800 mL distilled water and pour into a mixing container that is as close to the final volume as possible.
2. Add 10.4 g powdered RPMI medium (GIBCO) to 15 to 30 ^{o}C (room temperature) water with gentle stirring (Do not heat water).
3. Rinse out inside of package to remove all traces of powder
4. Add 34.5 g MOPS sodium salt (3-(N-morpholino) propanesulfonic acid), Fluka) into the medium
5. Dilute to a desired volume with water. Stir until dissolved.(Do not over-mix)
6. Adjust PH of medium to 0.2-0.3 below desired final working PH (7.0): use of 1N NaOH is recommended. (Add slowly with stirring). After pH has been adjusted. Keep container closed until medium is filtered
7. Sterilize immediately by 50 mm Diameter Filter Unit (150 mL or 250 mL, NALGENE) (Positive pressure recommended), and pH units usually rise 0.1-0.3 upon filtration and reach to 7.0
8. Label
9. Store @ 4 ^{o}C

Protocol 7. Preparation for 10% Glycerol/RPMI Buffer Stock Solution (100 mL)

1. Prepare 10% glycerol /RPMI buffer stock solution:

   90 mL RPMI buffer (RPMI 10.4 g/L H_2O, MOPS 34.5 g/L H_2O)

10 mL glycerol

Combine RPMI Buffer & Glycerol—VORTEX (for 2 min)

2. Filter sterilizes (0.45 microns)
3. Label
4. Store at 4 ^{o}C

Protocol 8. Inoculum Stock Solution (Long term)

1. Add 50 µL of inoculums to 1 mL RPMI Buffer/Glycerol (10% stock solution) in 5 mL round bottom tube
2. Vortex
3. Label and parafilm tube
4. Falcon Blue Max Jr., Falcon Max polypropylene Conical Tubes and Cryogenic vials can be used for storage at 4 ^{o}C
5. Select Isolate for Storage
6. From prepared conidial suspension pour off supernatant-add 5 mL of 10% Glycerol/RPMI Buffer stock spores (only)
7. Votex-pipette 0.5 mL conidial suspension into each cryogenic vial
8. Store at -80 ^{o}C

Protocol 9. Silica Gel Storage of Fungal Isolates (Long-term)

Using sterile technique, equipment and laminar flow food:

1. Shake gently but thoroughly the tube of milk suspension; flame the mouth of the tube.
2. Pour the milk suspension onto fungal growth in petri dish. Sometimes 2 tubes of milk suspension will be needed if the isolate exhibits a “fluffy” growth pattern.

3. Using a sterile glass rod, scrape the entire surface of the fungal culture to dislodge conidia – rotate dish gently to move milk over surface of the fungal growth.
4. Label the tube of silica gel to be used for storage; include the name of the isolate and the date (Example: Goff 9/10/96 skim or dry milk).
5. Remove screw cap from silica gel tube. Remove cotton stopper with sterile forceps, flame the mouth of the tube.
6. Using a sterile Pasteur pipette with a small pipette bulb attached, transfer 0.5ml – 2.0ml of the conidial-milk suspension into the silica gel tube. Flame the mouth of tube again; replace cotton stopper and screw cap securely.
7. Immediately Vortex. Place tube on Vortex mixer and allow shaking vigorously, turning the tube so that the conidial-milk suspension is mixed well onto the silica gel crystals. The tube of silica gel will begin to heat when the milk suspension makes contact with the crystals.
8. After the milk suspension has been combined well with the silica gel, plunge the tube into an ice bath to keep the contents from getting hot enough to kill the fungus. Be sure the tube has the cap screwed tightly to prevent moisture from entering the tube.
9. After the tubes have cooled in the ice bath they may be stored in the refrigerator for an indefinite period of time.
10. To start a fresh fungal culture from silica gel storage – shake out a few crystals onto prepared medium (PDA or Oatmeal-PDA) in petri dishes and allow fungus to grow.

Protocol 10. Target Range for %T (625 nm) for Conidia Concentrations

	BECKMAN		SHIMADZU	
Fungus	Target Range for %T (625 nm)	%T for $1\times10^{\wedge6}$ CFU/mL	Target Range for %T (625 nm)	%T for $1\times10^{\wedge6}$ CFU/mL
B. cinerea	8 --- 62	39	27 --- 79	60
C. acutatum	2 --- 79	56	20 --- 83	64
C. fragariae	15 --- 70	55	30 --- 77	67
C. gloeosporioides	37 --- 72	61	50 --- 78	69
C. gloeosporioides 162	34 --- 65	55	51 --- 77	68
F. oxysporum	30 --- 65	55	49 --- 78	70
P. obscurans	46 --- 80	77	50 --- 77	75
P. viticola	37 --- 69	6	48 --- 76	67

*Inoculum must be within the ranges showed on the table for bioautography and microtiter assay. The target range for %T is the ideal point for fungus growth; CFU=Colony Forming Units.

Protocol 11. McFarland 0.5 Barium Sulfate Turbidity Standard

1. McFarland Standard consists of 50uL 0.048mol/L $BaSO_4$ stock solution and 9.95 mL 0.18 mol/L H_2SO_4 stock solutions.
2. H_2SO_4 stock solution: 0.18 mol/L H_2SO_4 (stock solution: 1mL conc. H_2SO_4 + 99 mL H_2O.
3. Barium chloride stock solution: 0.048mol/L BaCl stock solution (1.175g BaCl*2 H_2O: 99mL H_2O).

4. 0.5 McFarland standard % T (625 nm) = 70%
5. Goes bad: make up every 3months.

Protocol 12. Spotting and Spraying TLC plates for Bioautography

Make 20 mg/mL of test samples: weigh the test samples and then adding the corresponding solvents.

1. Label with date, fungal isolate, and name and/or sample designation on the top of the plate.
2. For each essential oil treatment add 4 uL and 8uL onto different lineated sections on the plate.
3. Spray fungal spore solution on the plate; remember to hold the plate as flat as possible, silica side up (spray once and allow to dry then completely cold the plate in the spore/ media solution and second time before placing directly into chamber).
4. Place in moisture chamber and then into 250 ^{o}C incubator for 4 days.
5. Measure the diameter of inhibitory zones and make any other observations you can about the plates.

Protocol 13. Procedures for Grape Media

Wash and rinse grape leaves (20 leaves) with DDI H_2O. Allow the leaves to drain (paper towel) to remove excess water. Using a Waring Commercial Blender grind Grape leaves (with 500 mL DDI H_2O). Pure the suspension into 500 mL erlenmeyer flask with a stir bar in it. Stir (Sti/Hot plate-**No Heat**!) until completely blended. Check pH of media (pH of Grape broth is around 3.0) and adjust it until 6.5 using 1 M NaOH. Wrap mouth of flask with folded aluminum foil and seal with autoclave tape. Sterilize at 121 °C for 45 minutes.

Protocol 14. Procedures for Agar Media

20 g Bacto Agar

1000 mL DDI H_2O

Using a 1000 mL erlemeyer flask combine agar and water. Stir (Stir/Hot plate) and heat until completely blended. Divide media into 500 mL aliquots in 1000 mL Erlenmeyer flask (to allow for volume during autoclaving). Wrap mouth of flask with folded aluminum foil and seal with autoclave tape. Sterilize at 121 for °C 45 minutes.

Protocol 14. Procedures for Strawberry Agar Media

500 mL Grape Media

500 mL Agar media

Using cooled sterilized Grape media pour into flask containing cooled agar media. Combine media and reseal flask with sterile aluminum foil removed from containing Agar media. Mix them well and pour plates in sterile hood while media is warm. Do not allow media to get cold.

Protocol 15. Murashige and Skoog Supporting Solution

1. M&S Basal Salt Mixture (Sigma M5524) 2.2 g/L
2. Sucrose (Sigma S8501) 10 g/L
3. Gamborg's Vitamins 5 mL (of a 100x prepared solution) /L
4. pH 5.7
5. Phytagel (Sigma P8169) 1 g/L

Protocol 16. MES with Sucrose

1. (2-[N-Morpholino] ethane sulfonic acid) hydrate (Sigma M8250) 1 mM in water

2. Sucrose (Sigma S-8501) 2% W/V

3. pH 6.5

4. Phytagel (Sigma P8169) 1 g/L

Protocol 17. Hoagland Solution

1. Hoagland Basal salts (Phytotechnology Laboratories, P.O. Box 13481, Shawnee Mission, KS 66282 3.2 g/L

2. Phytagel (SigmaP8169) 1 g/L

Protocol 18. Preparation of Difco Mycological Broth

Yeast	40 g
Mycological Broth	100 g
Sucrose	400 g
DDI H_2O	2 L
pH	4.8

Heat and stir

Protocol 19. Preparation of Shredded Wheat Medium

200 mL	Difco mycological broth
100 g	shredded wheat
put in 2.0 L	Fernbach flask

Autocalve for 45 min @ 121 °C

Protocol 20. Preparation of 2% Malt Extract Agar (MEA) Medium

200 mL of 2% MEA medium:

Malt extract 2% × 200 = 4 g

Peptone 0.1% × 200 = 0.2 g

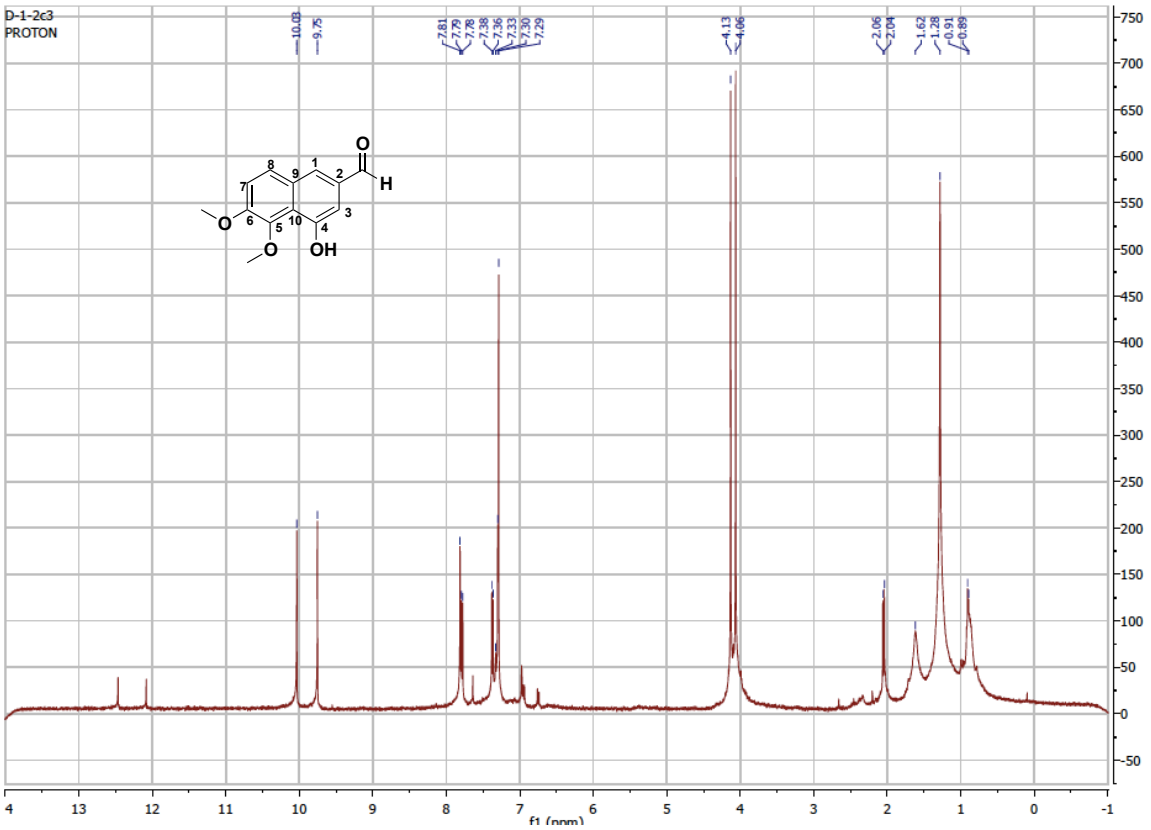
Glucose 2% × 200 = 4 g

Agar 2% × 200 = 4 g

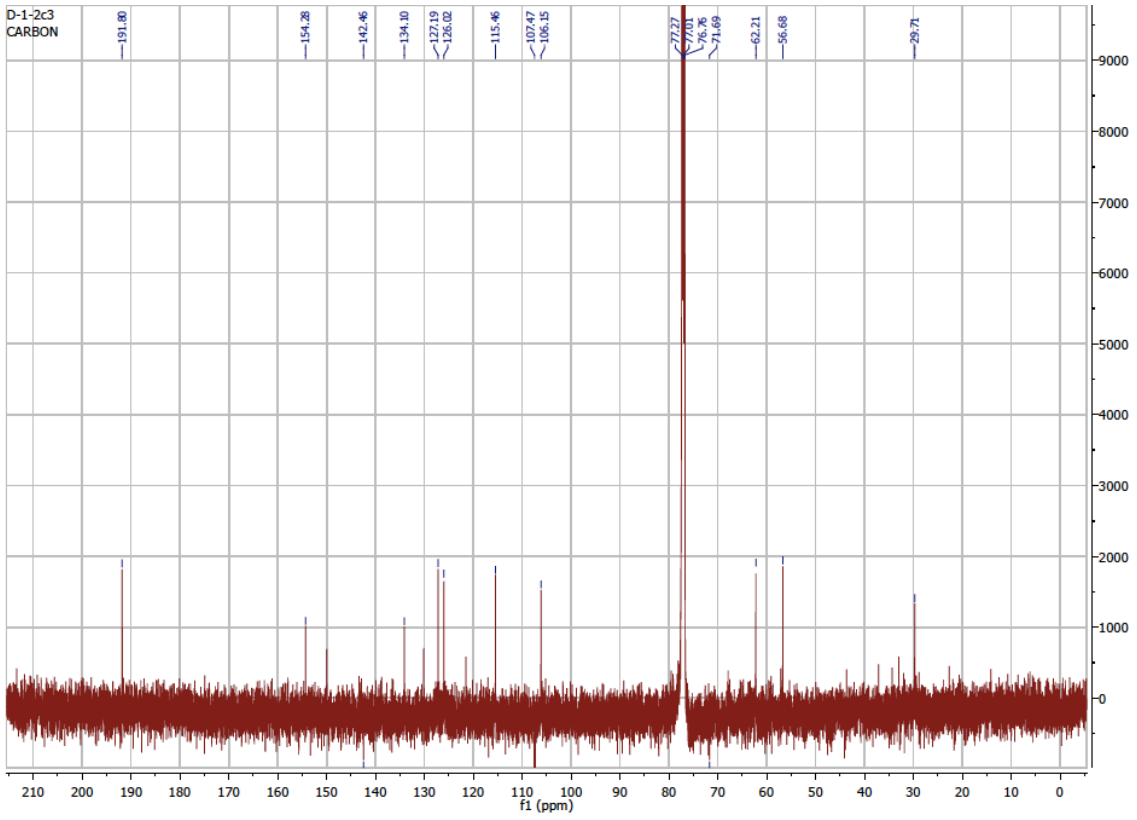
APPENDIX: B

1D and 2D NMR and UV Data of the Pure Compounds

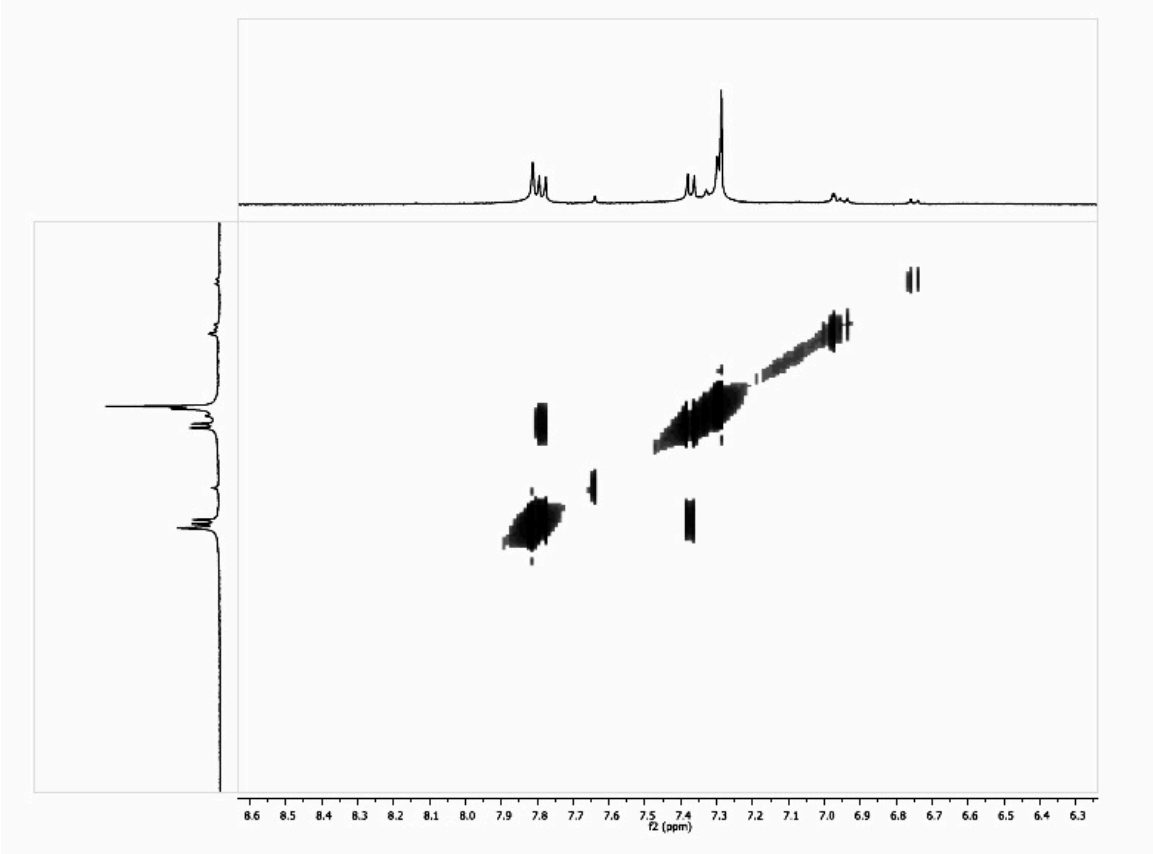
^{1}H NMR spectrum of 4-hydroxy-5, 6-dimethoxy-2-naphthaldehyde (**9**)



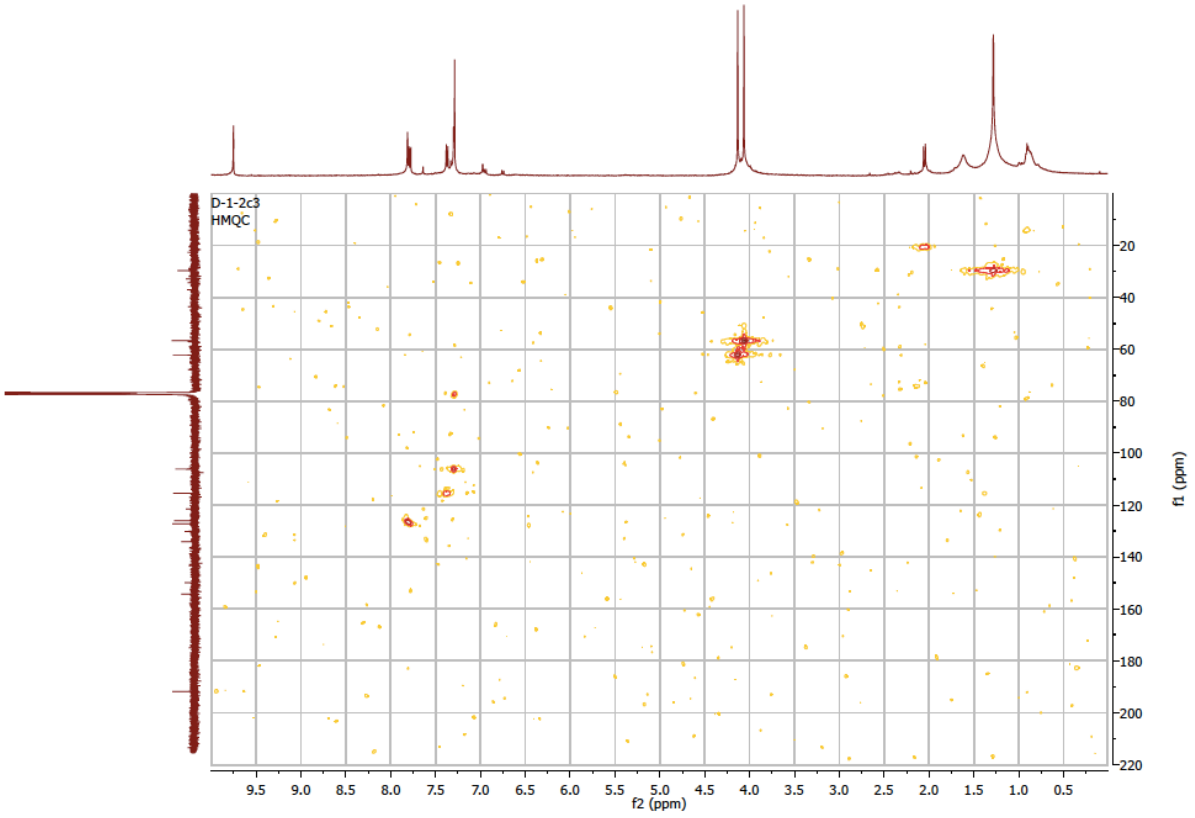
^{13}C NMR spectrum of **9**



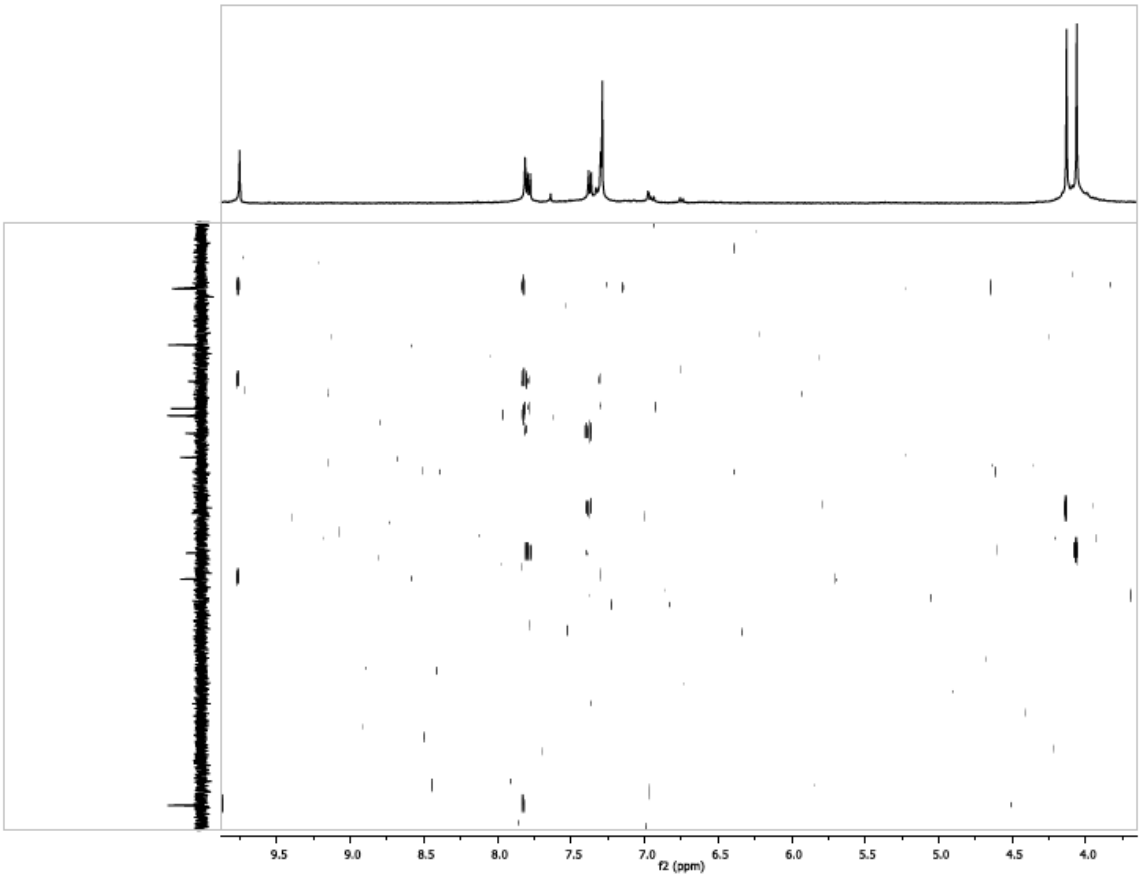
COSY spectrum of **9**



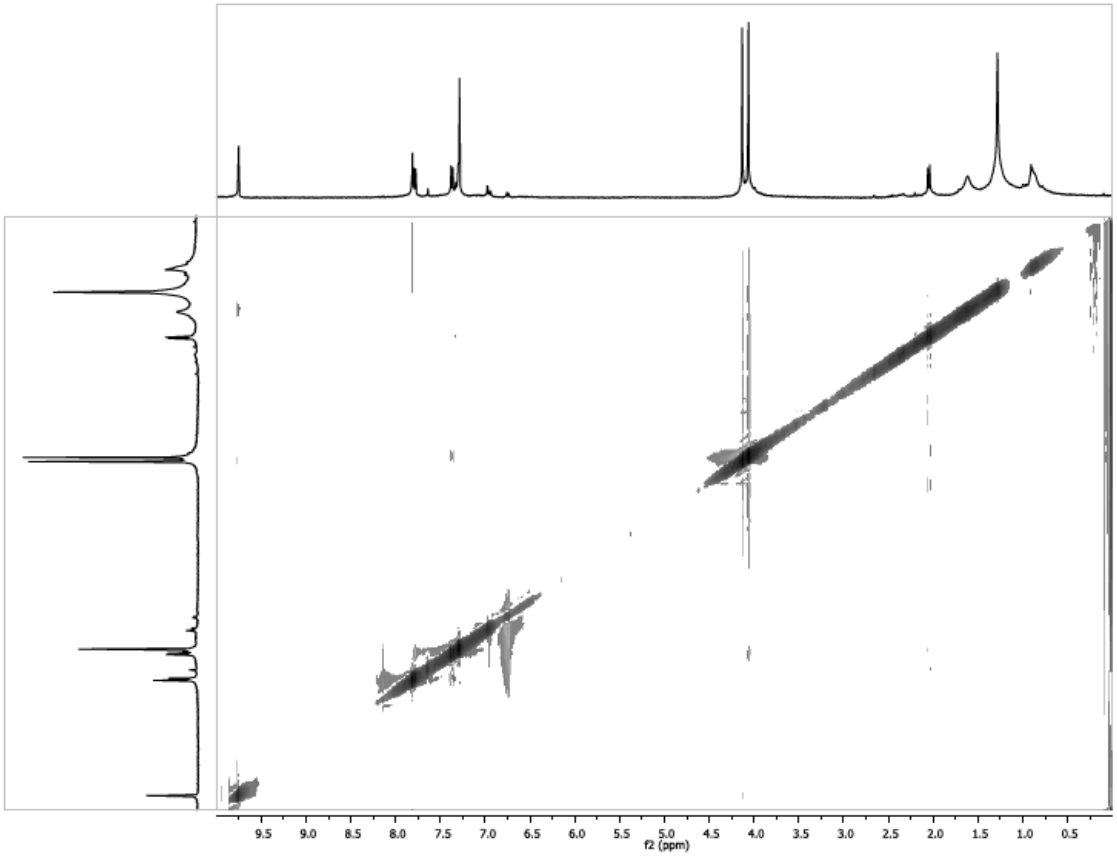
HMQC spectrum of **9**



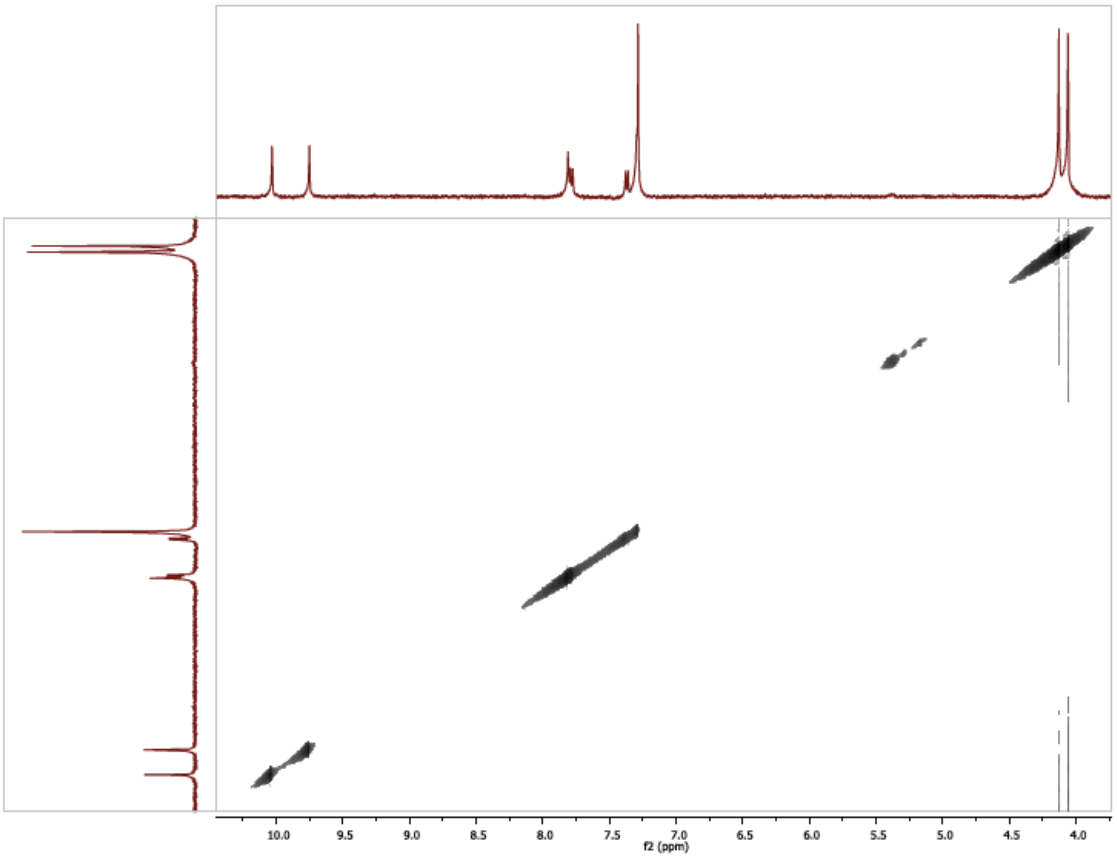
HMBC spectrum of **9**



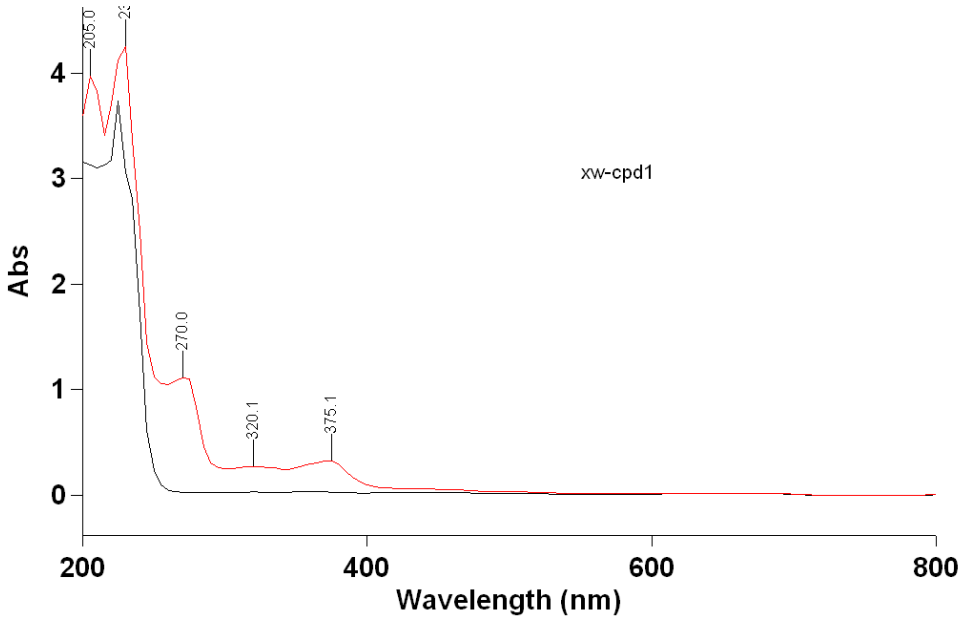
NOESY spectrum of **9**



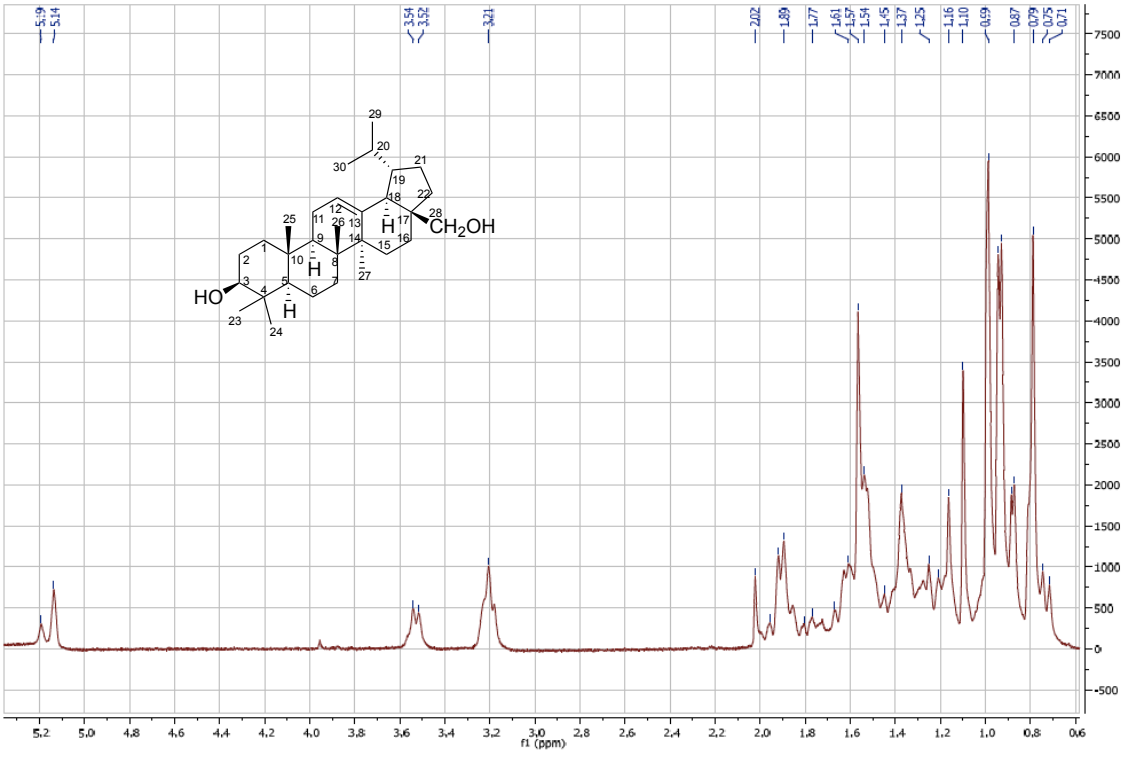
ROESY spectrum of **9**



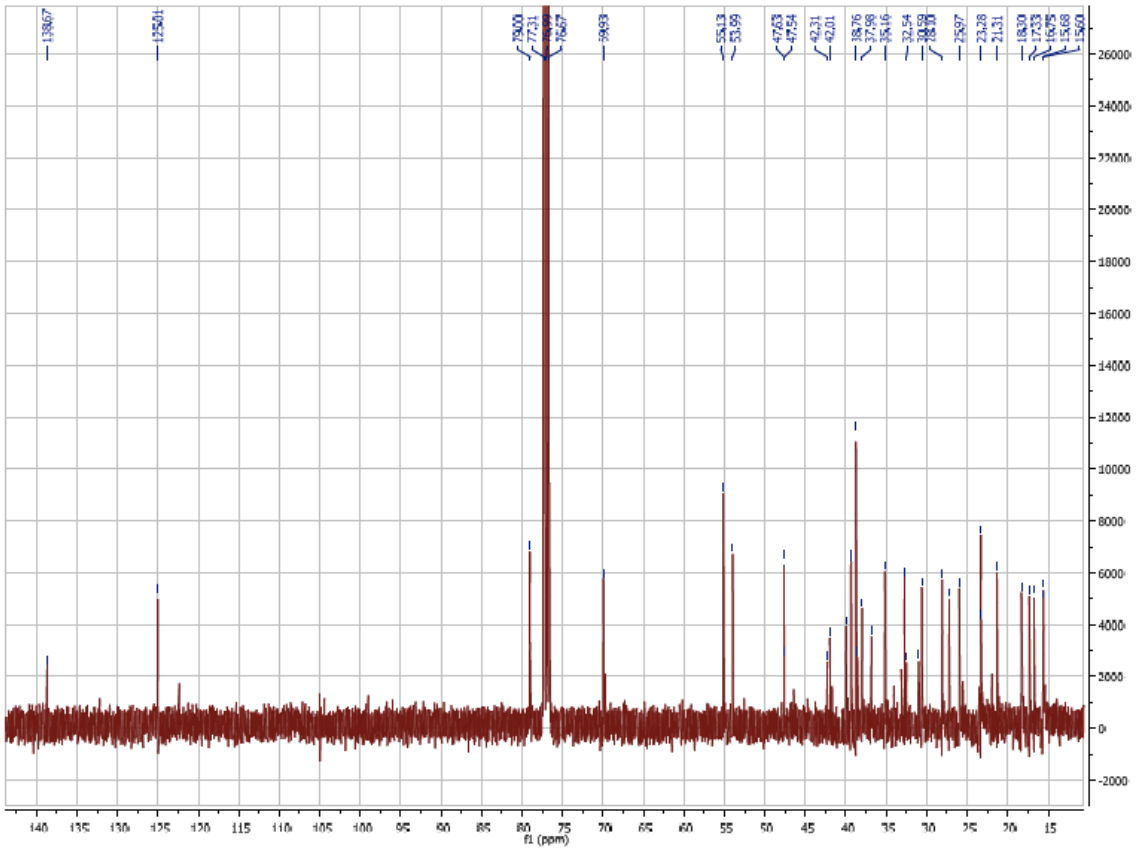
UV of **9**


205.0
270.0
320.1
375.1
xw-cpd1
Abs
Wavelength (nm)
0
1
2
3
4
200
400
600
800

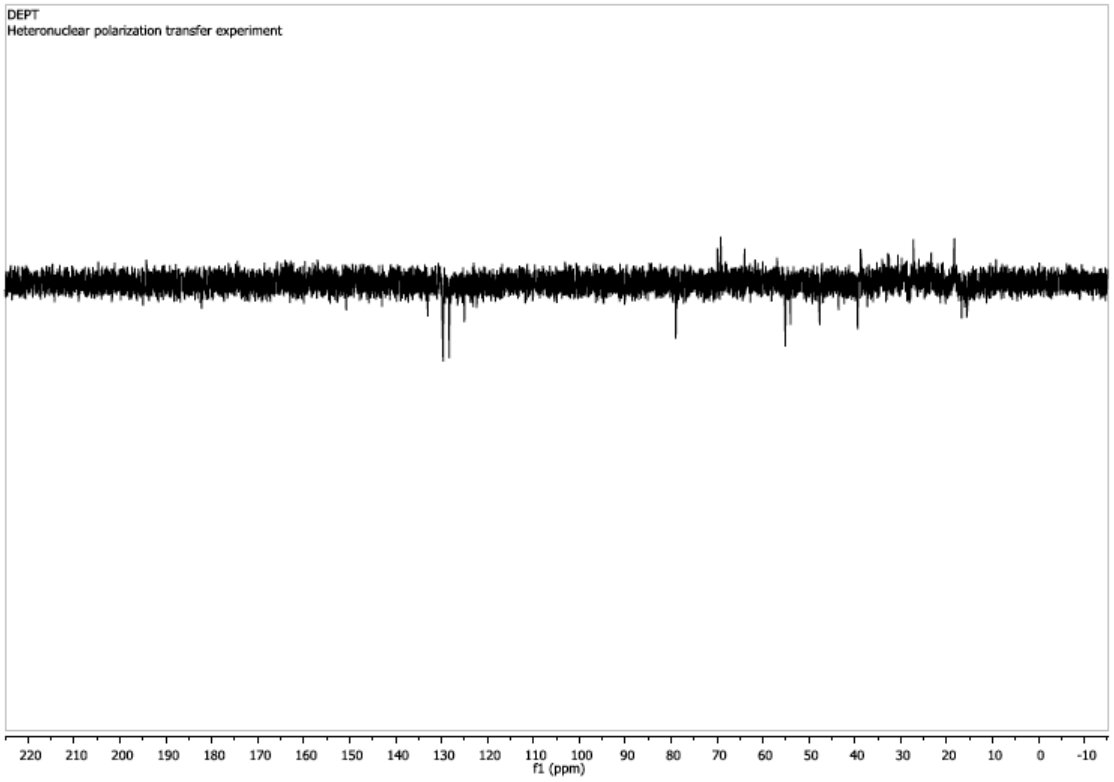
^{1}H NMR spectrum of 12, 13-didehydro-20, 29-dihydrobetulin (**10**)



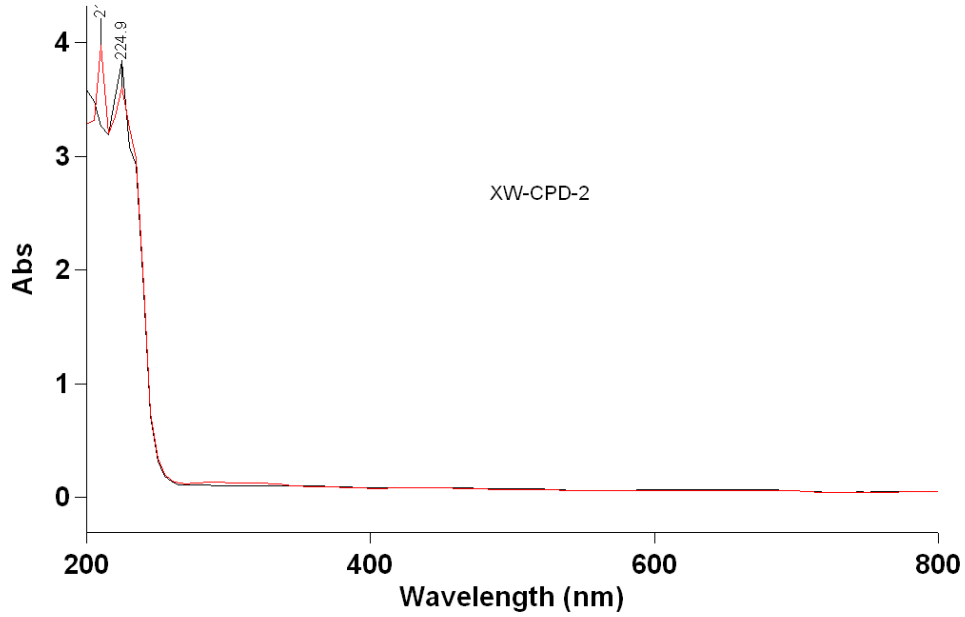
^{1}C NMR spectrum of **10**



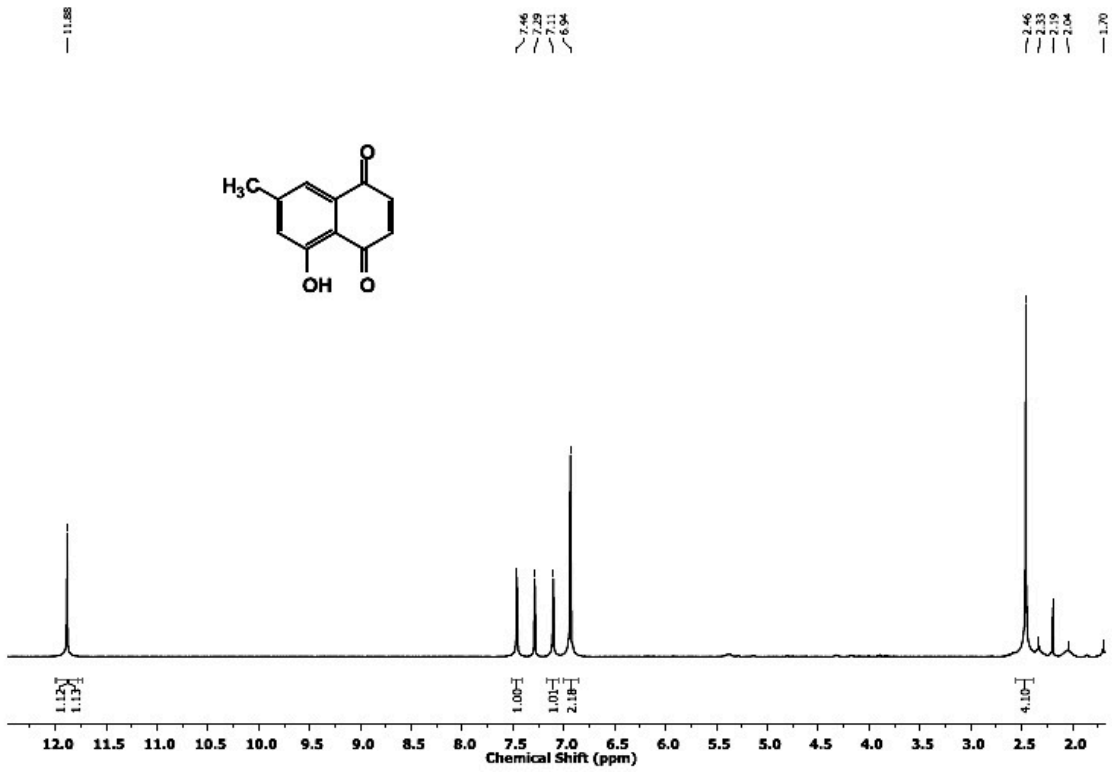
DEPT spectrum of **10**



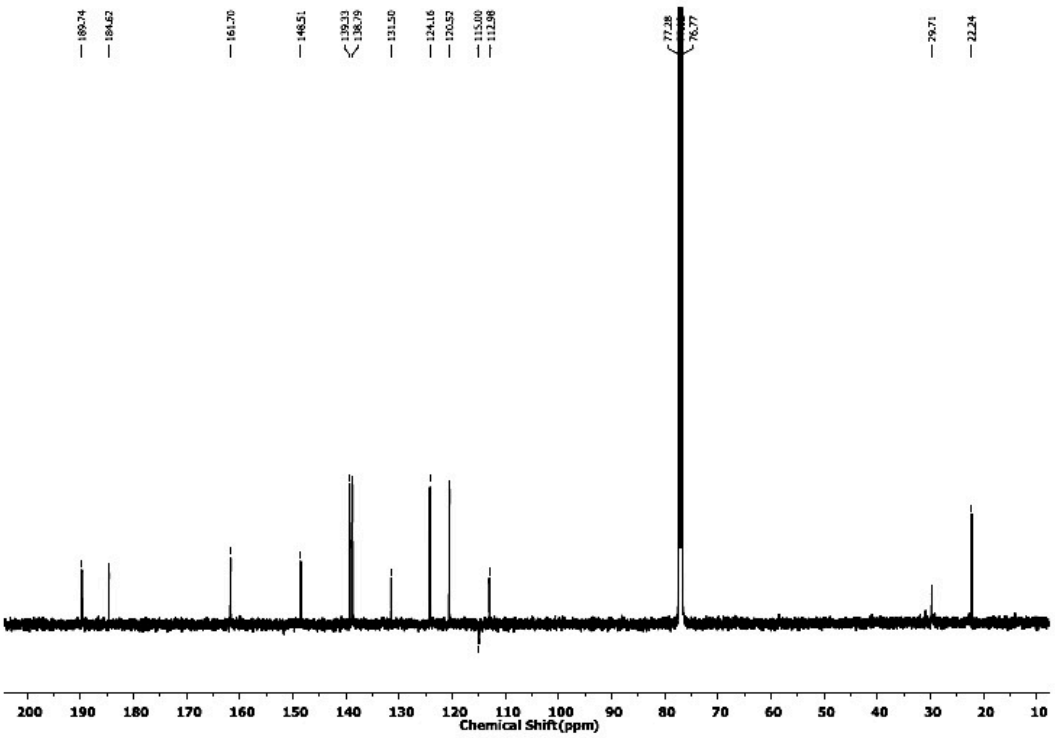
UV of **10**



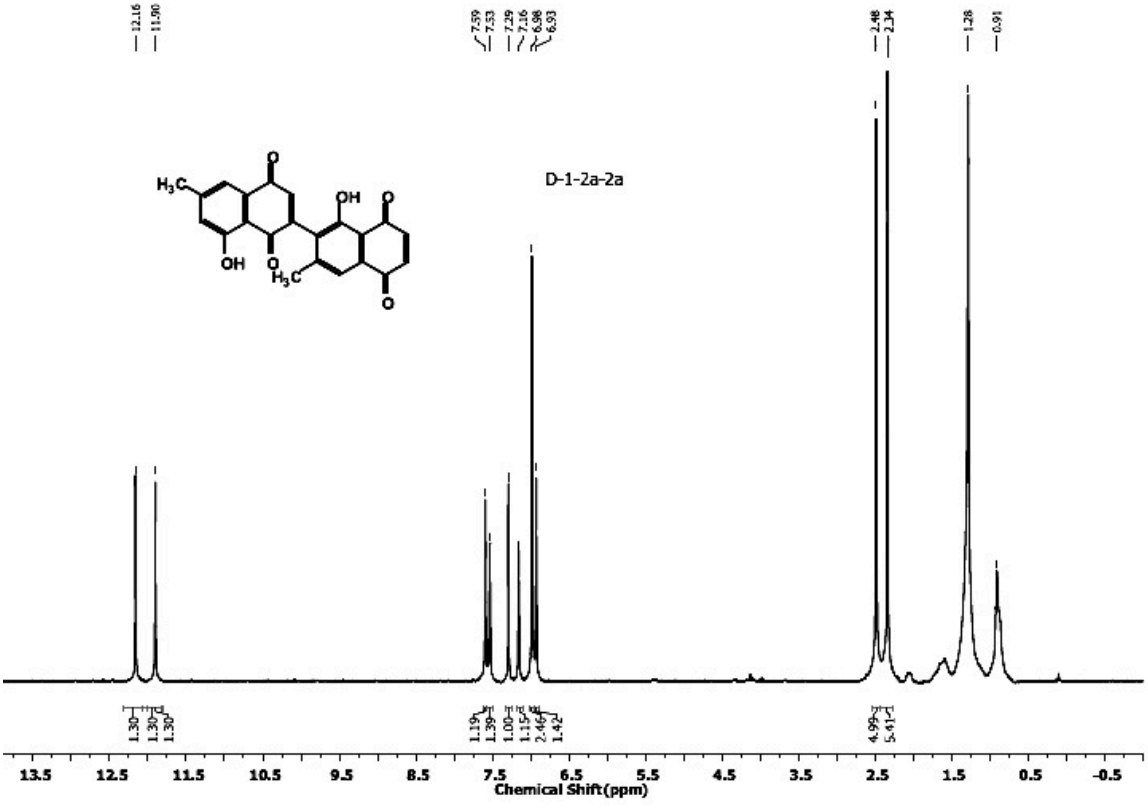
^{1}H NMR spectrum of 7-methyljuglone (**11**)



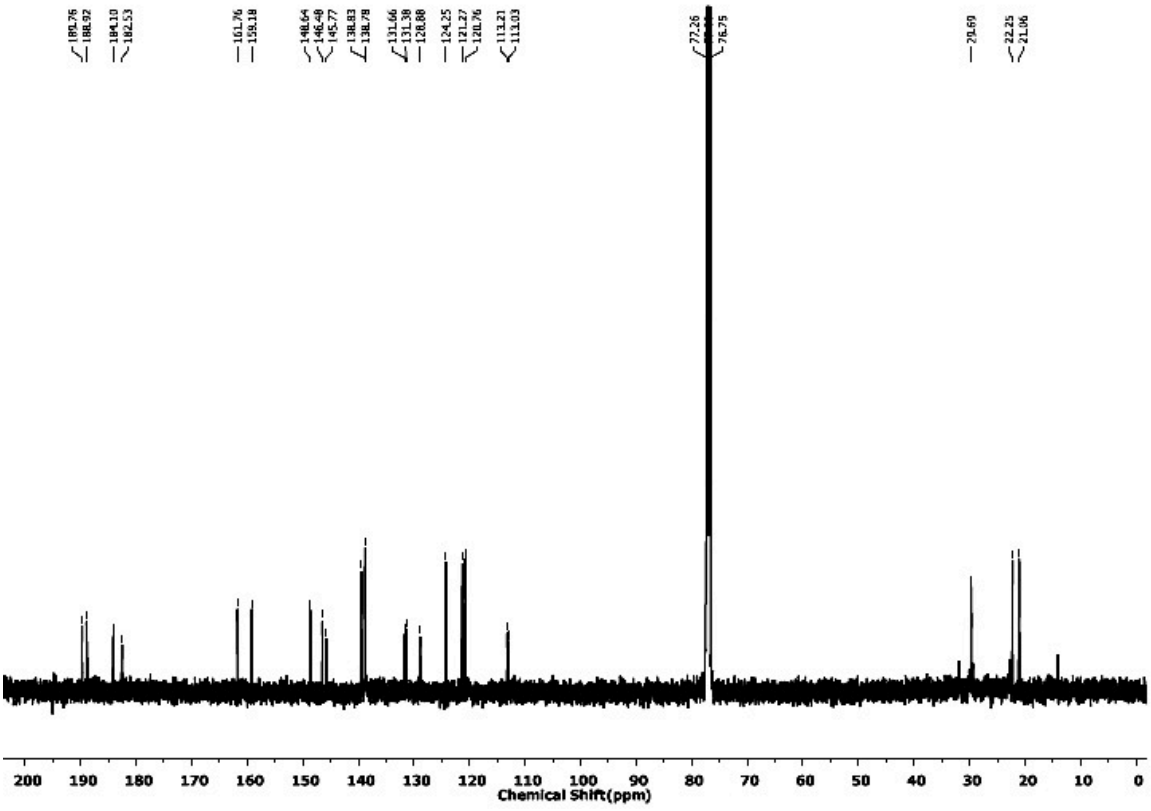
^{13}C NMR spectrum of 11



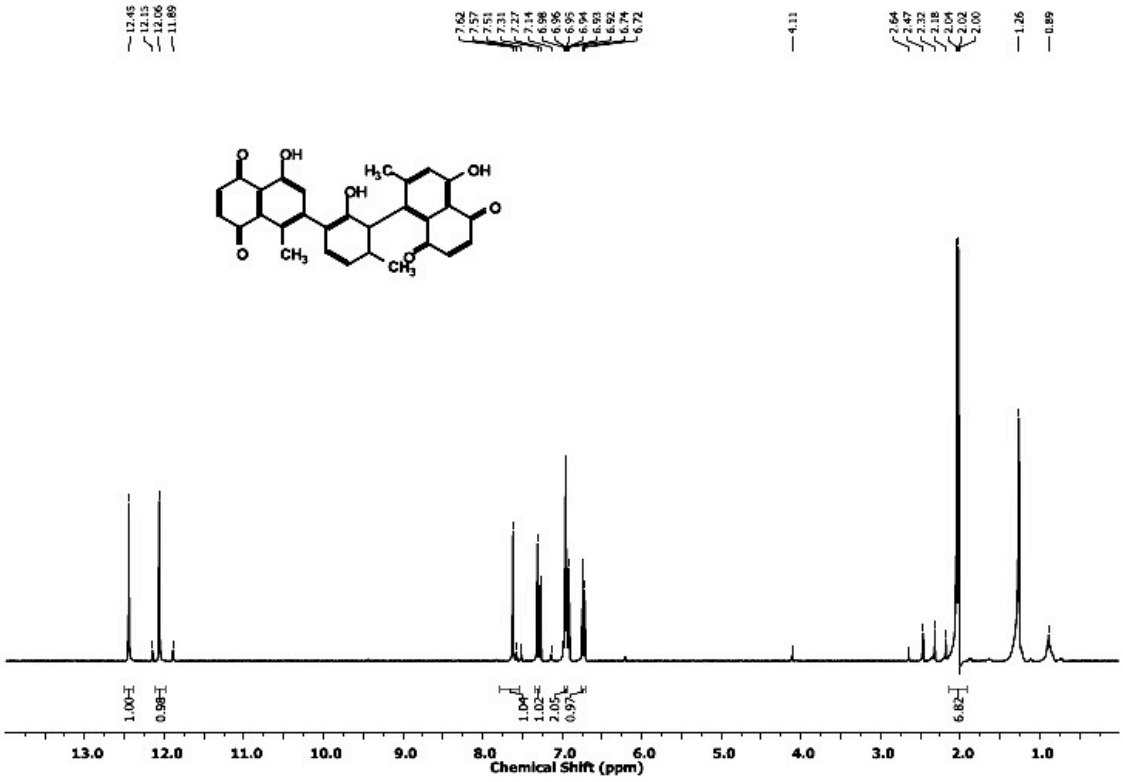
^{1}H NMR spectrum of diospyrin (**12**)



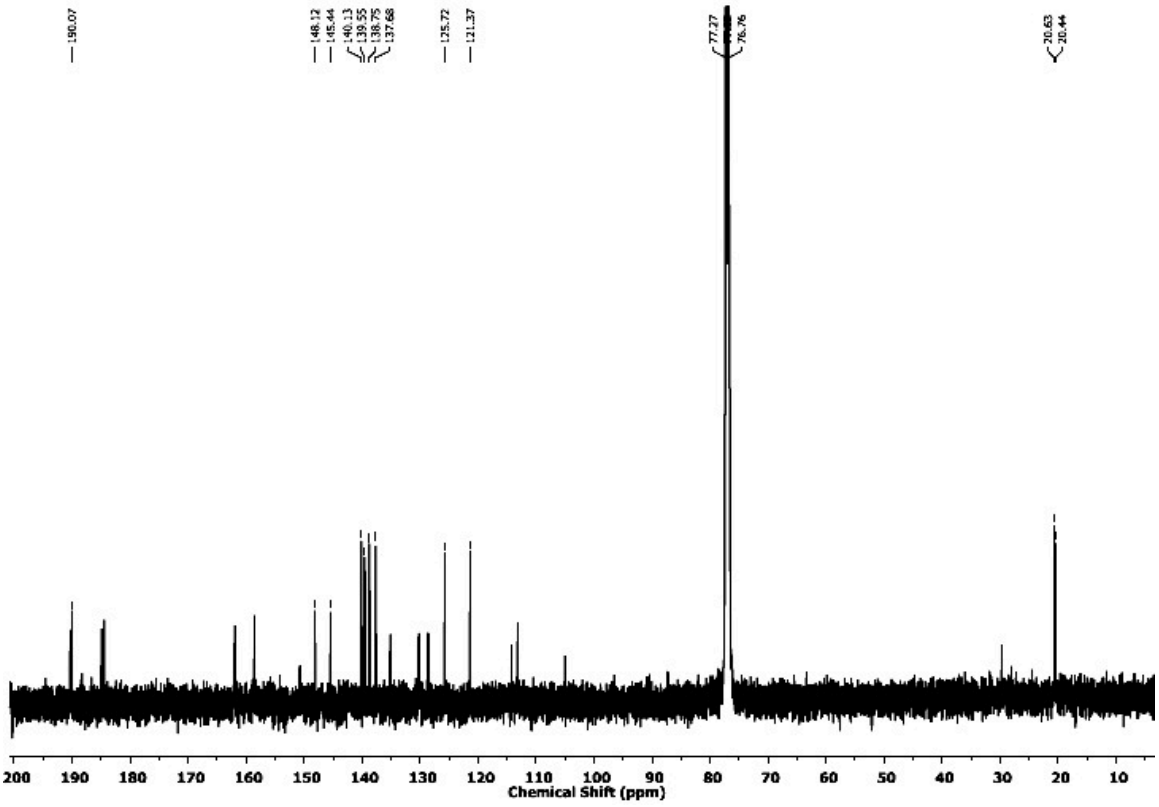
^{13}C NMR spectrum of **12**



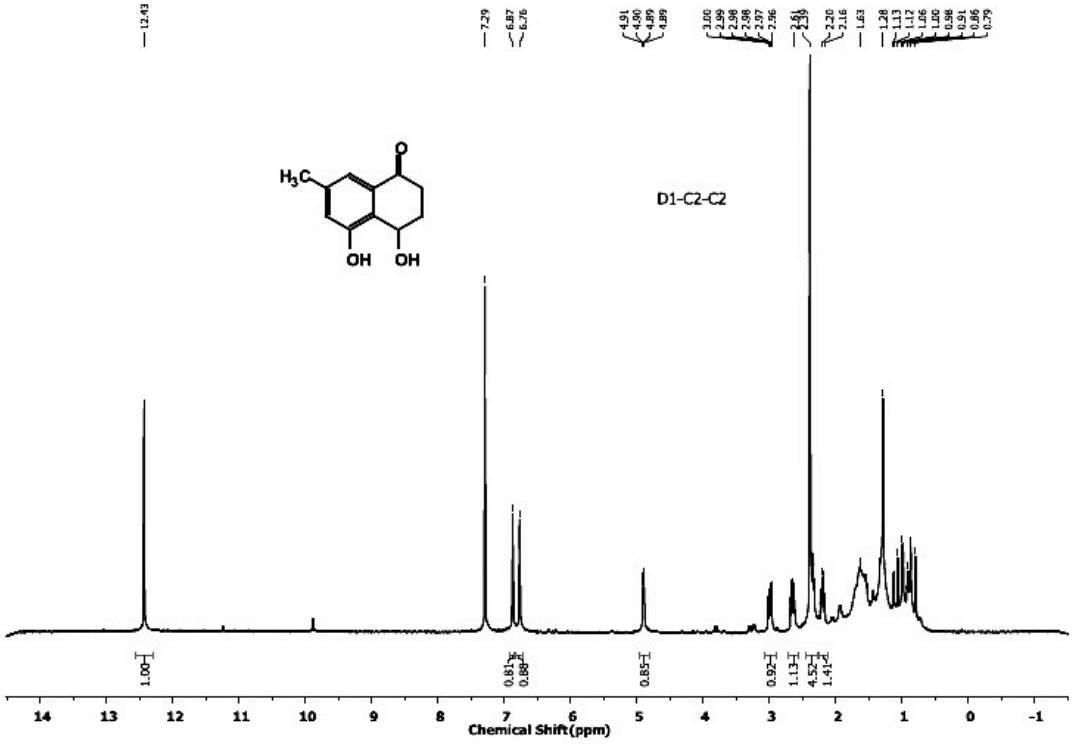
^{1}H NMR spectrum of isodiospyrin (**13**)



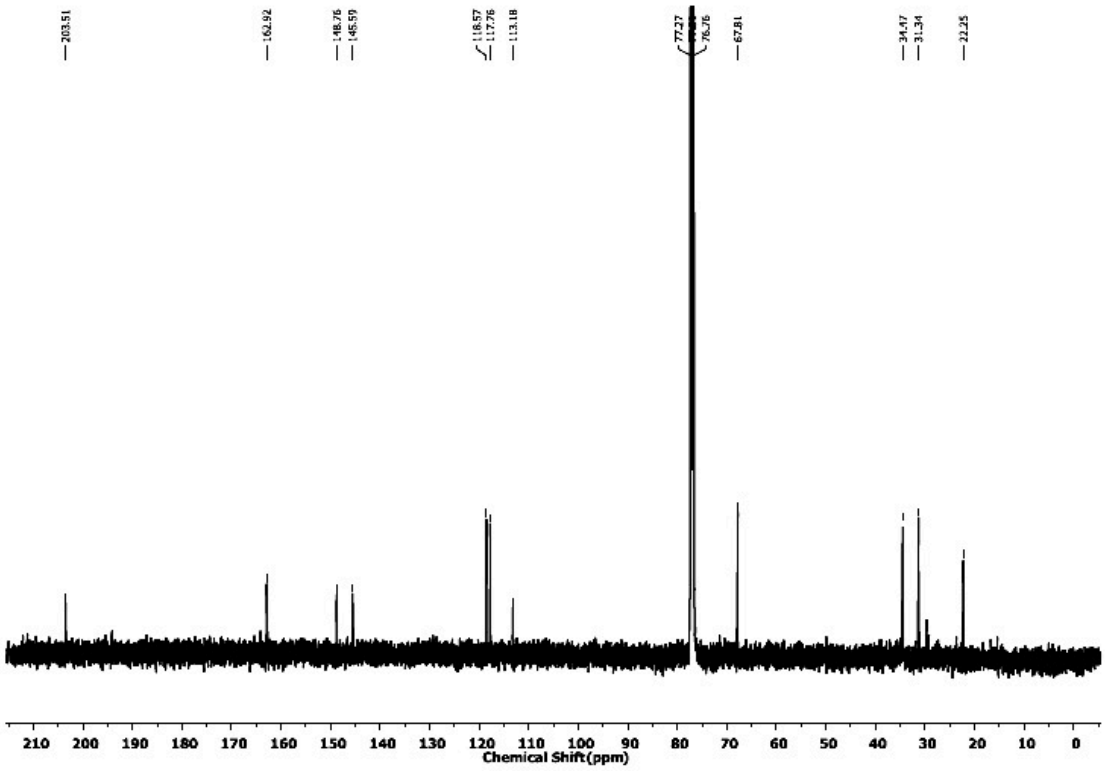
^{13}C NMR spectrum of **13**



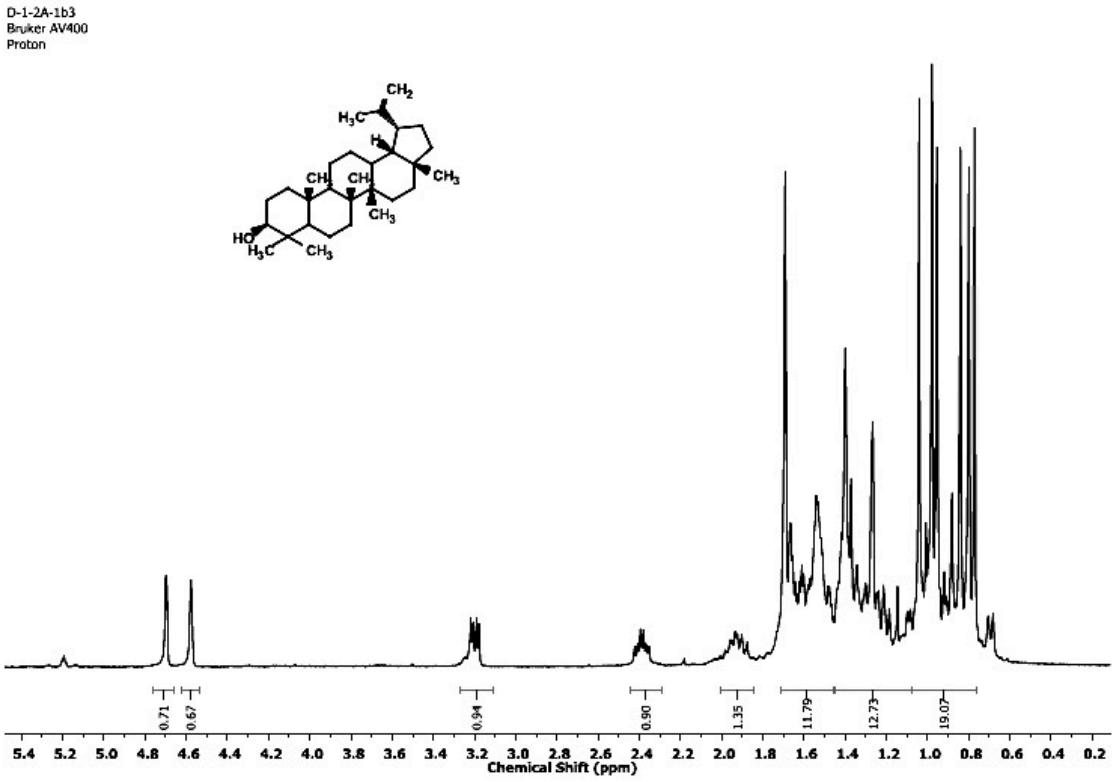
^{1}H NMR spectrum of shinanolone (**14**)



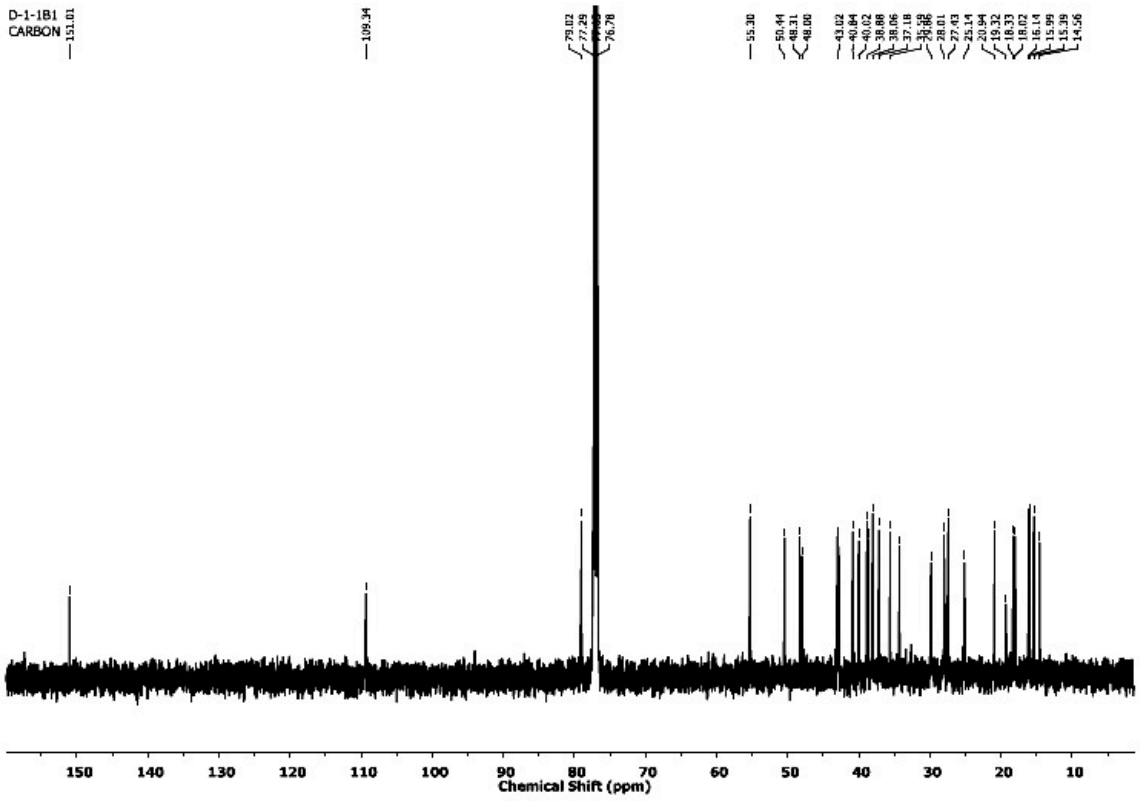
^{13}C NMR spectrum of **14**



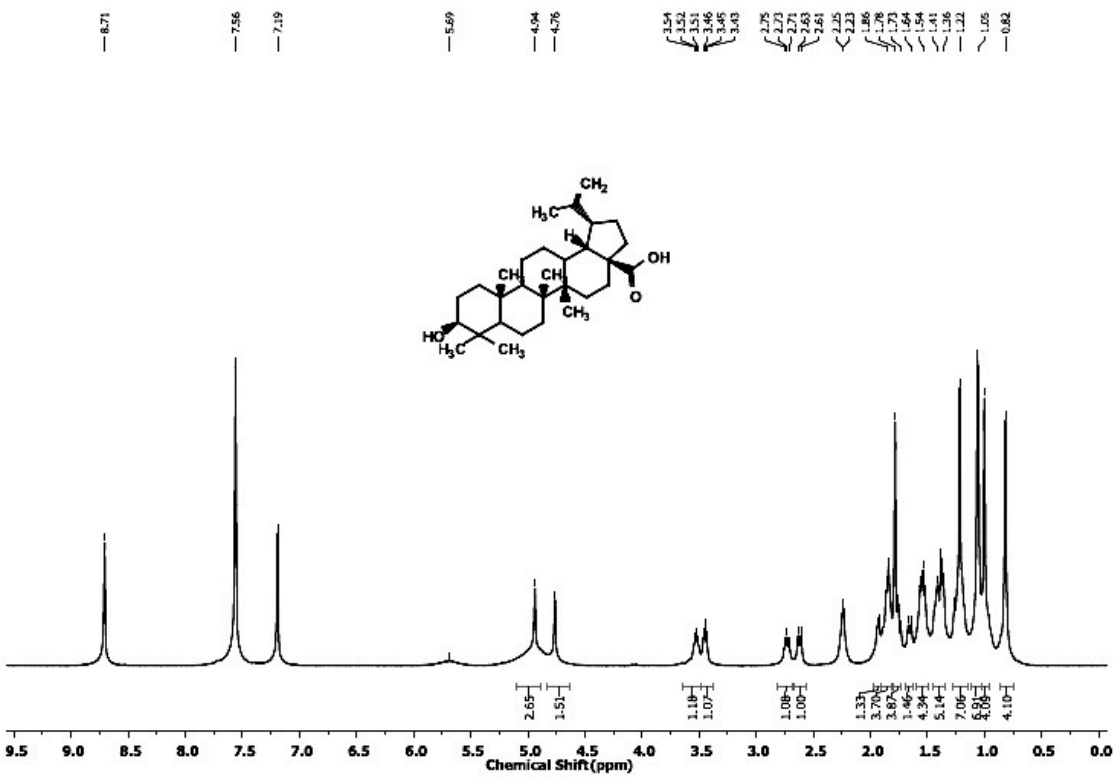
^{1}H NMR spectrum of lupeol (**15**)



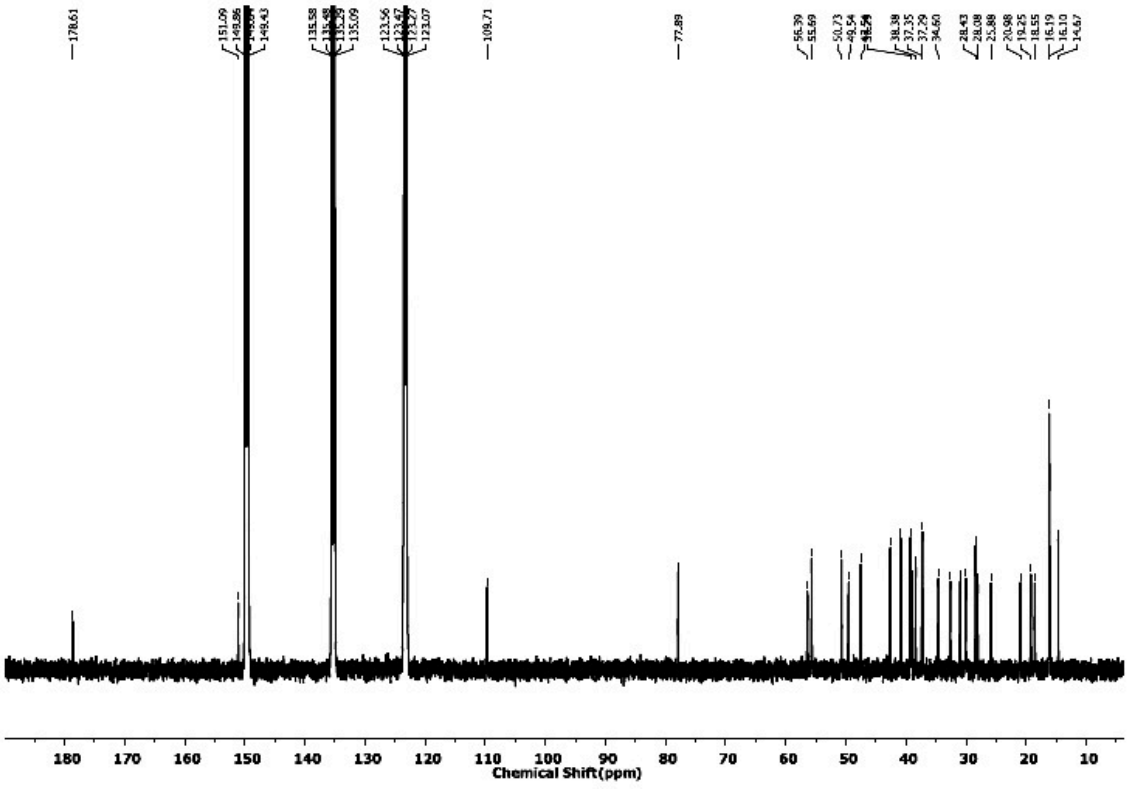
^{13}C NMR spectrum of **15**



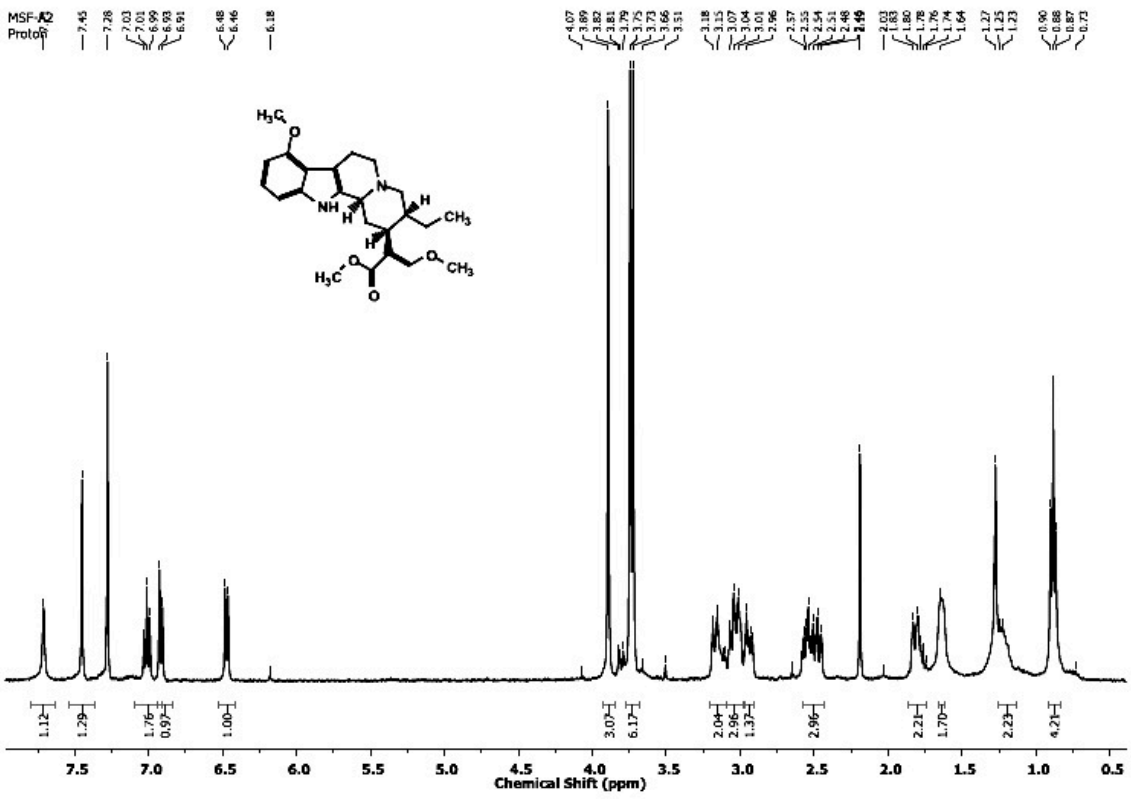
^{1}H NMR spectrum of betulinic acid (**17**)



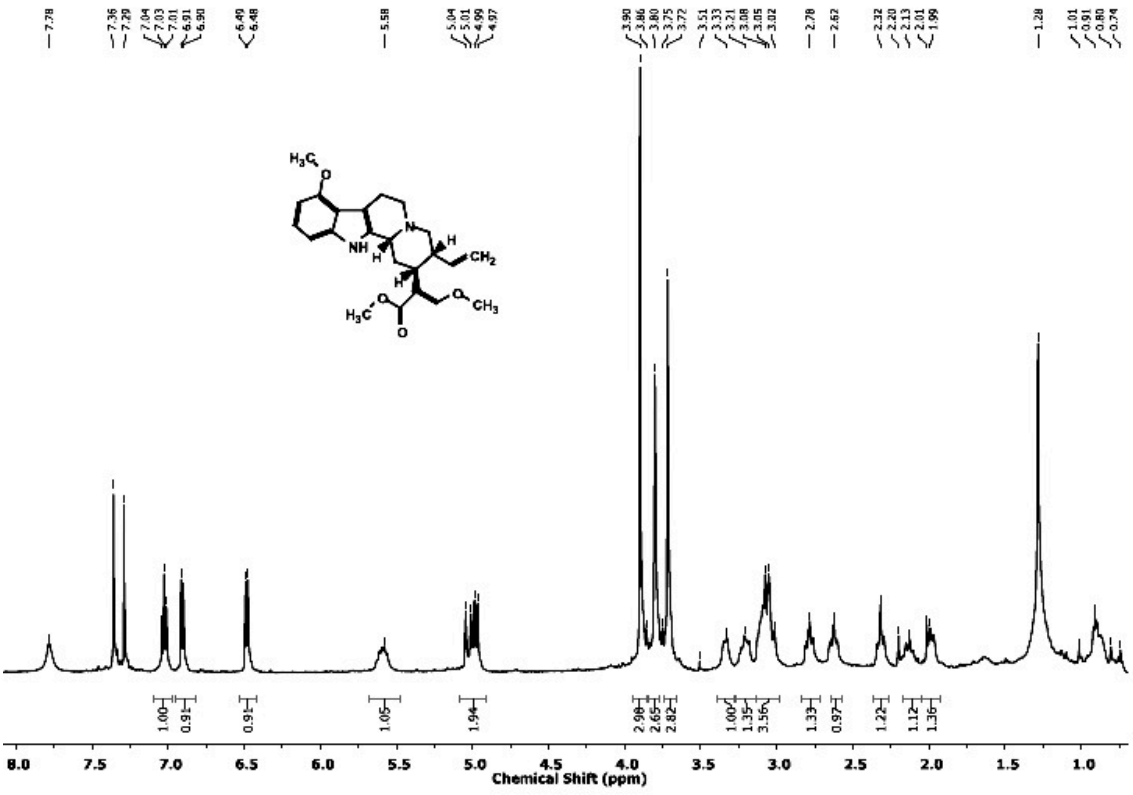
^{13}C NMR spectrum of **17**



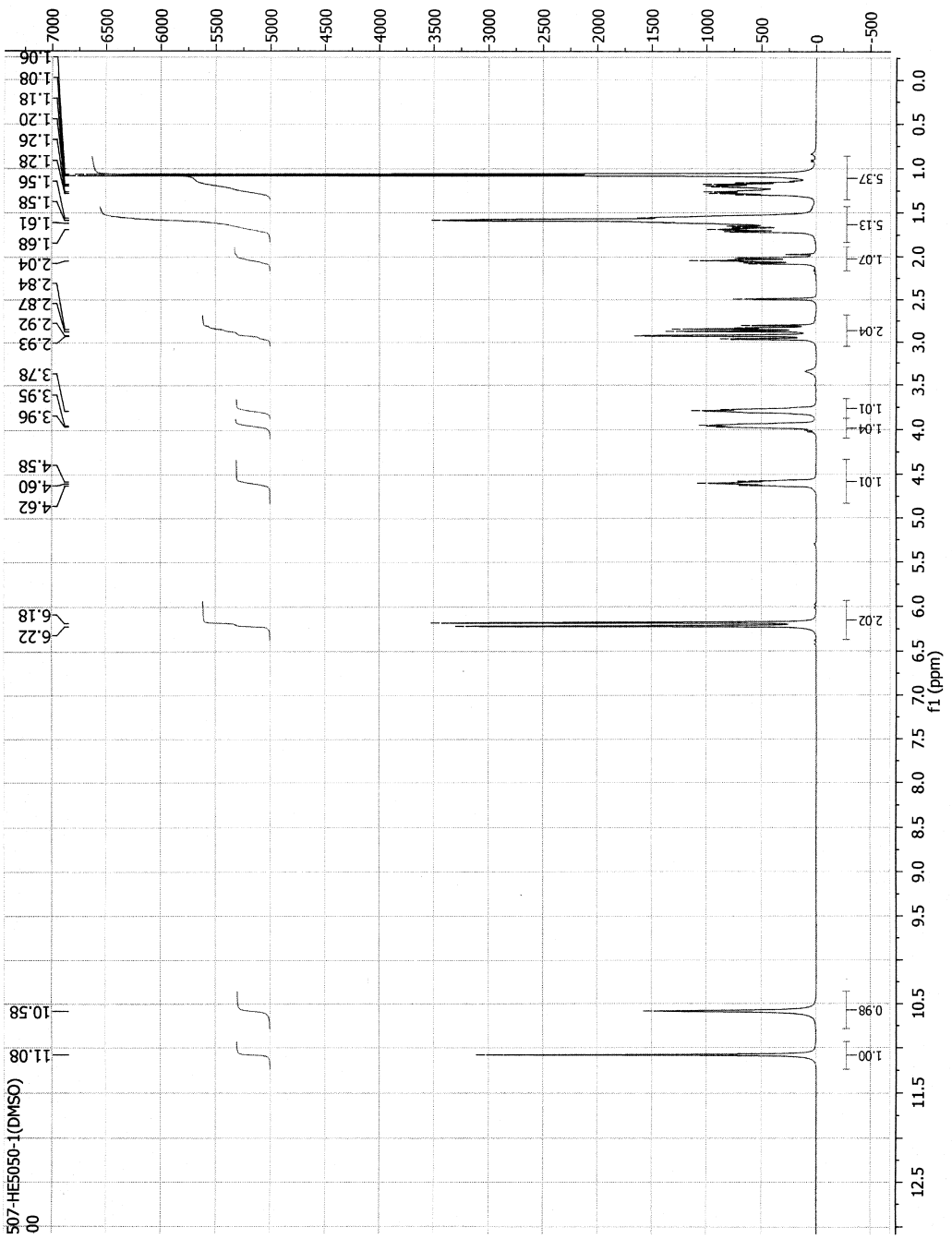
^{1}H NMR spectrum of mitragynine



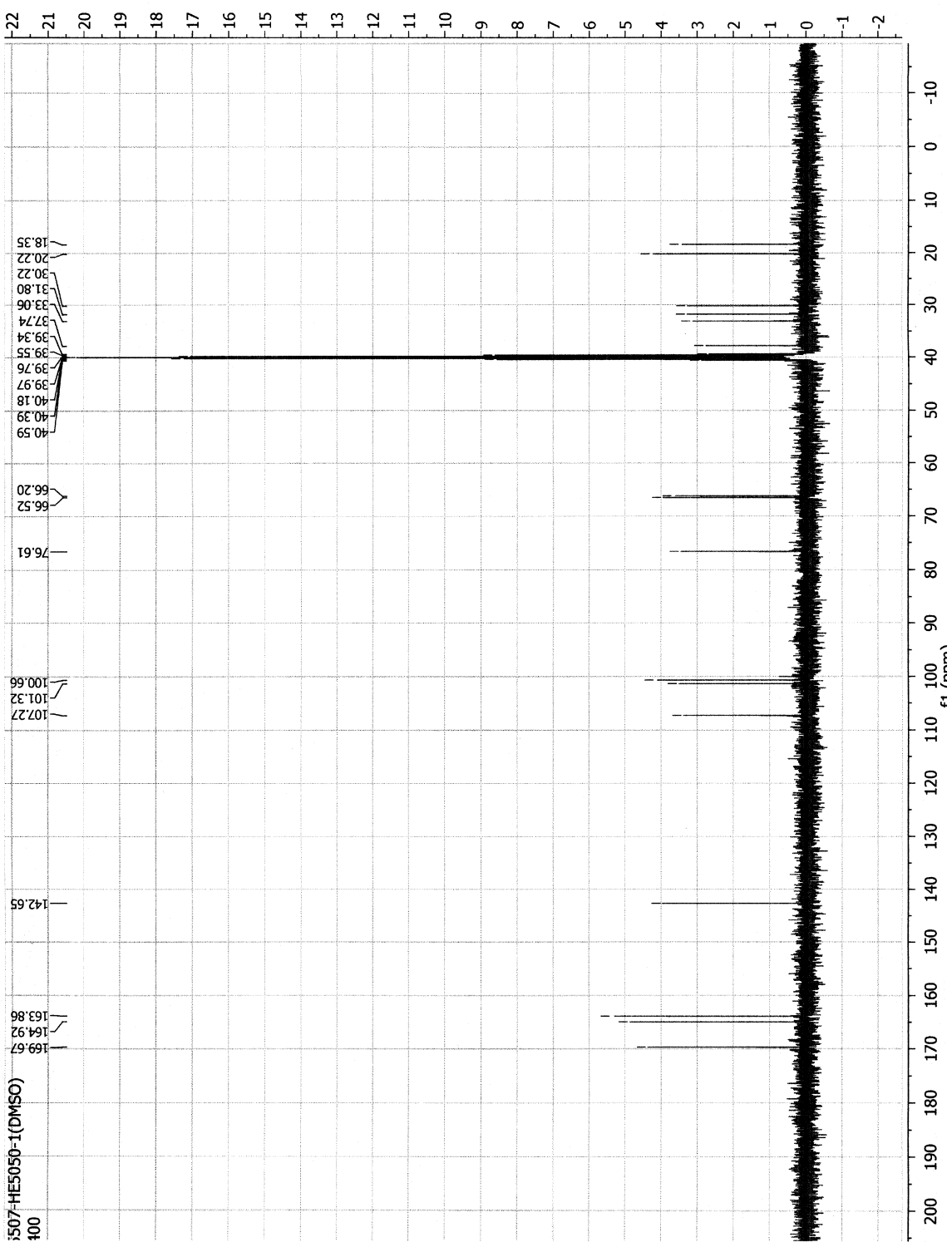
^{1}H NMR spectrum of paynantheine



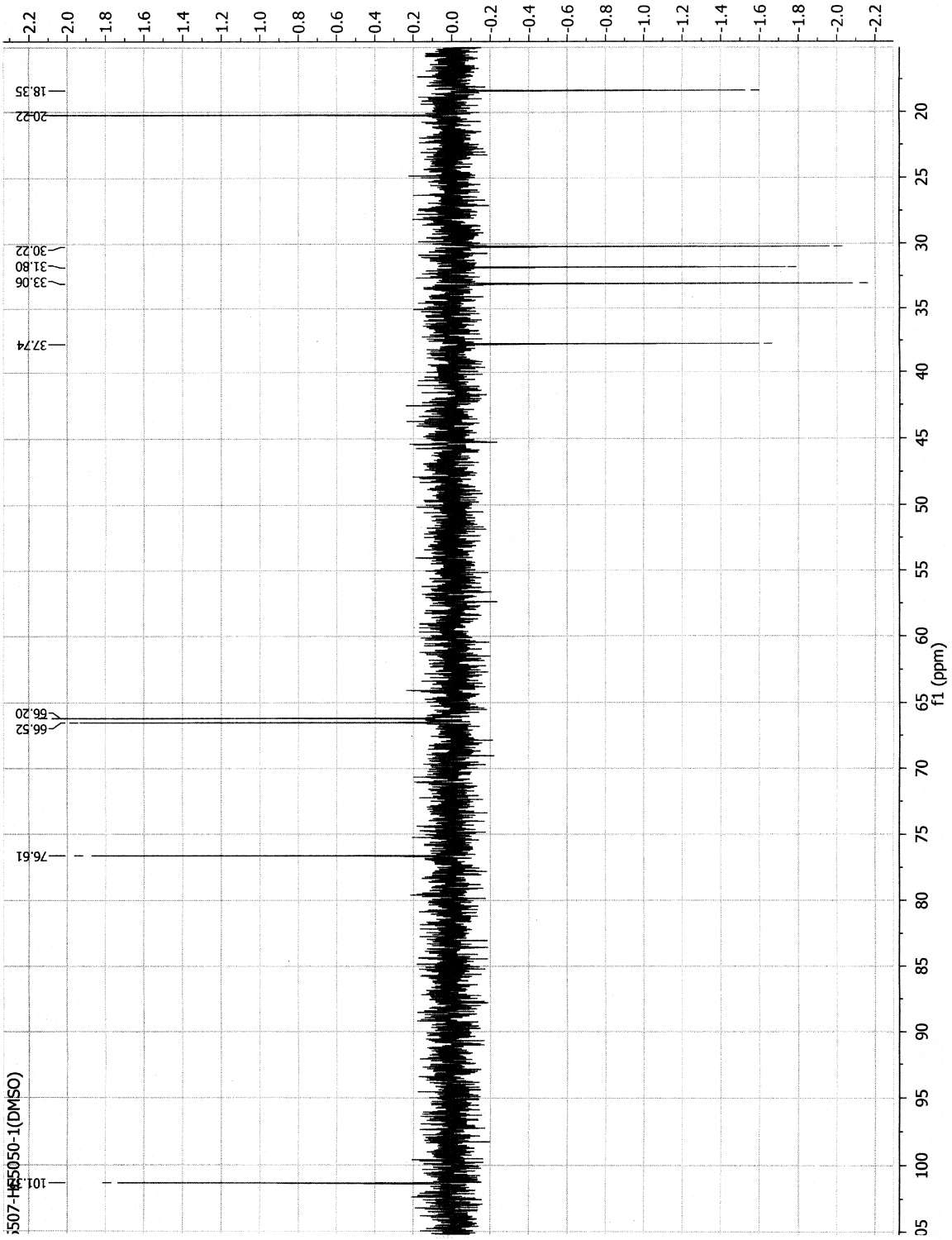
^{1}H NMR spectrum of cladosporin (**20**)



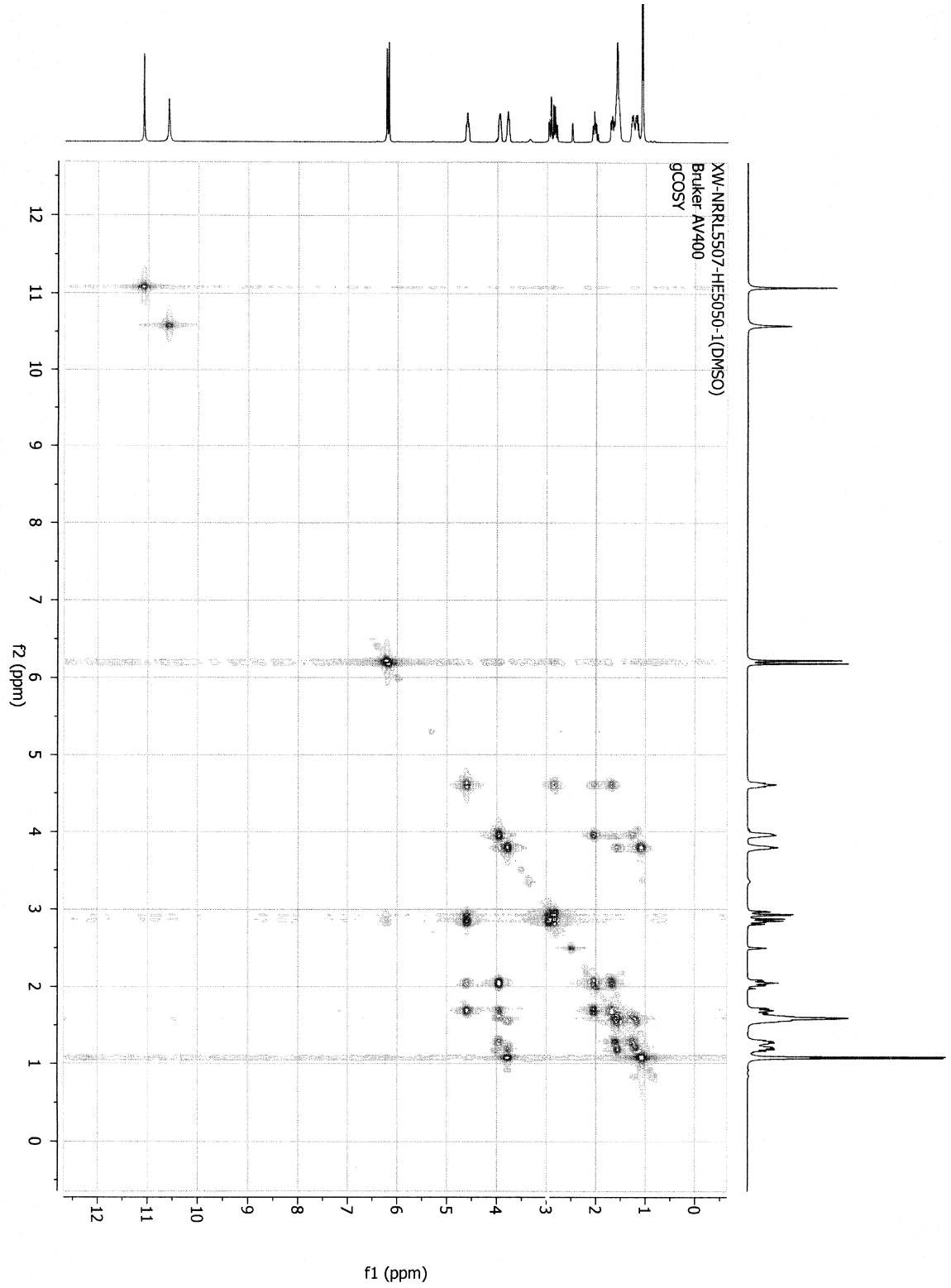
^{13}C NMR spectrum of **20**



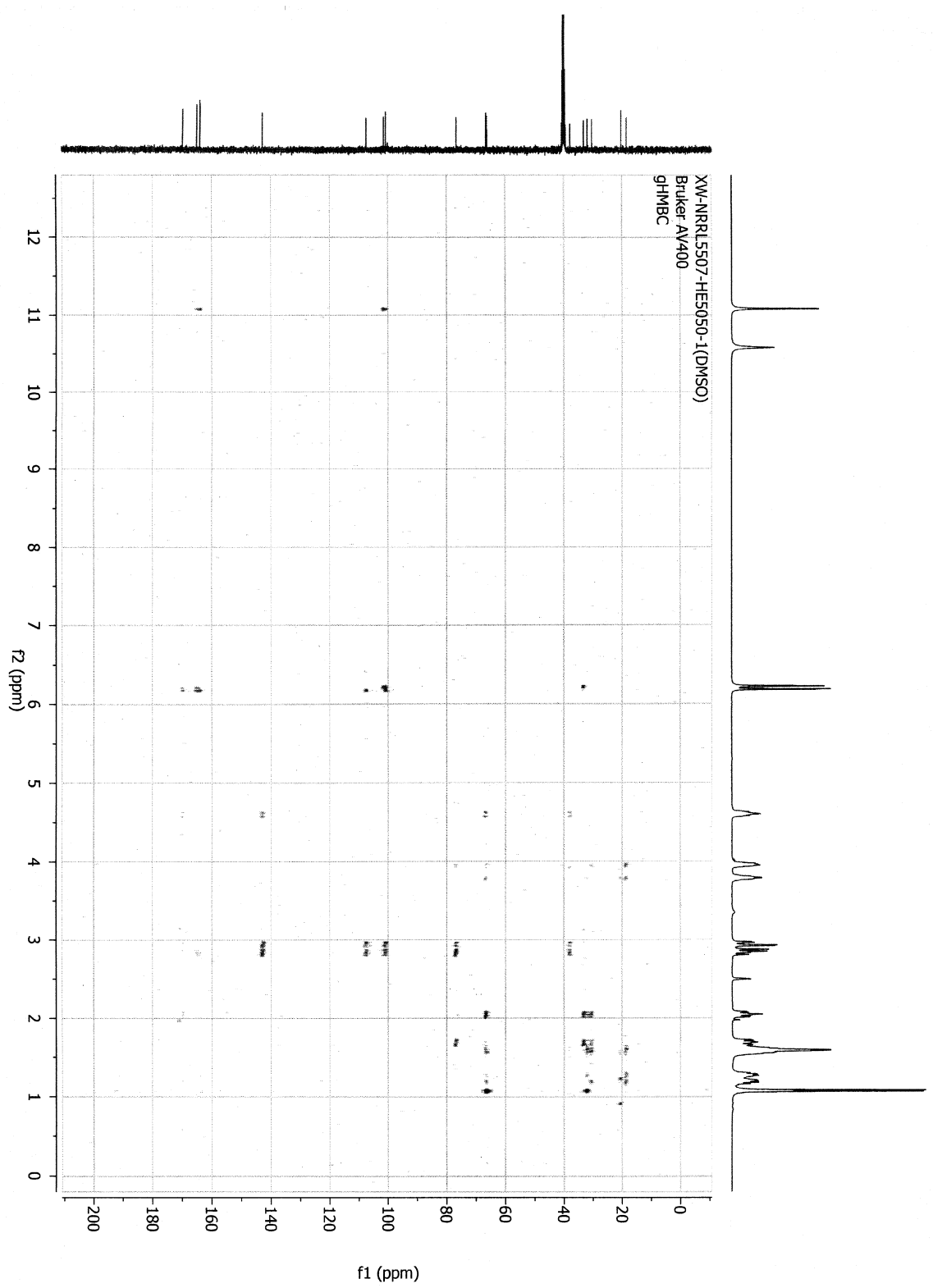
DEPT spectrum of **20**



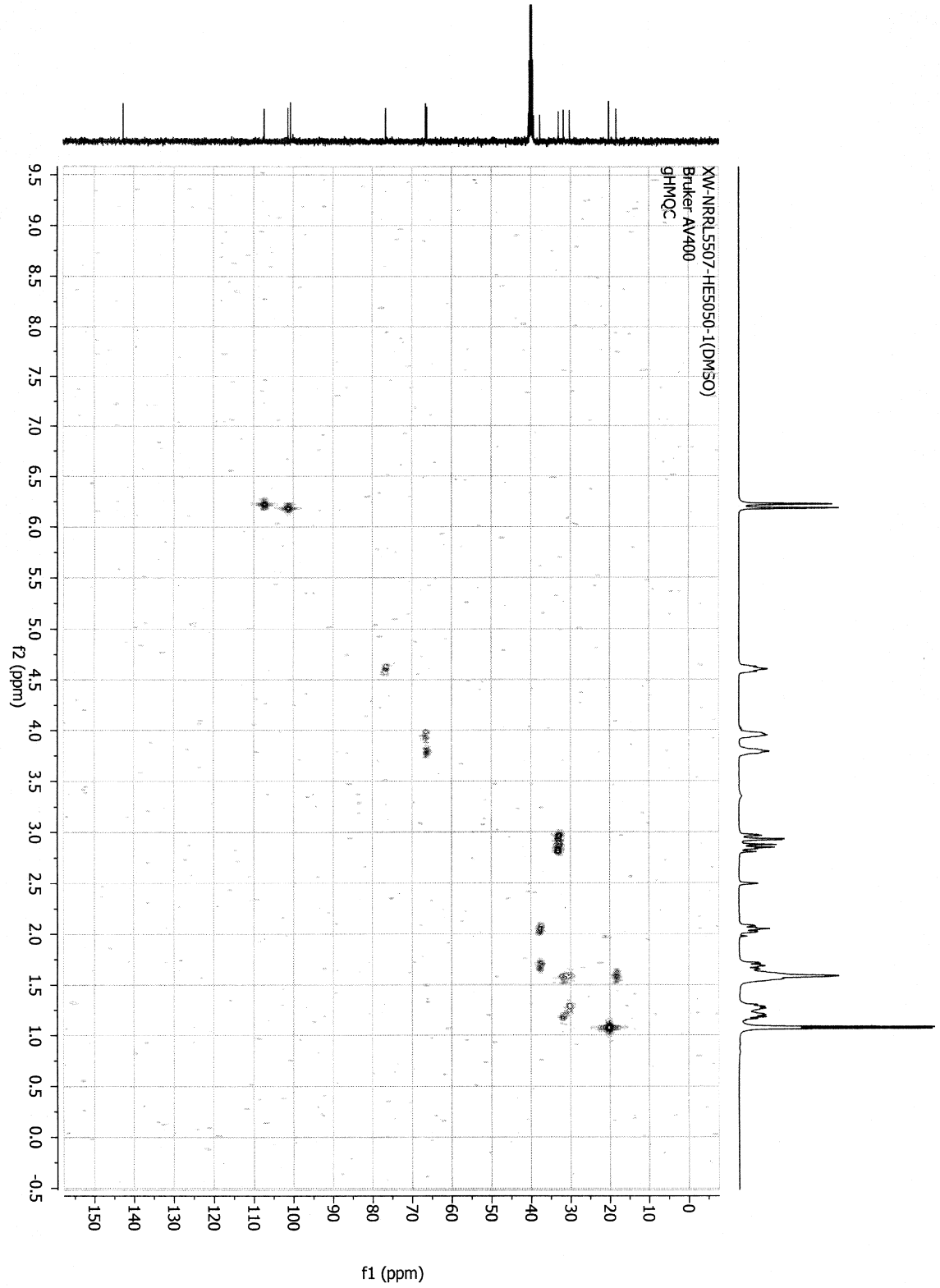
COSY spectrum of **20**



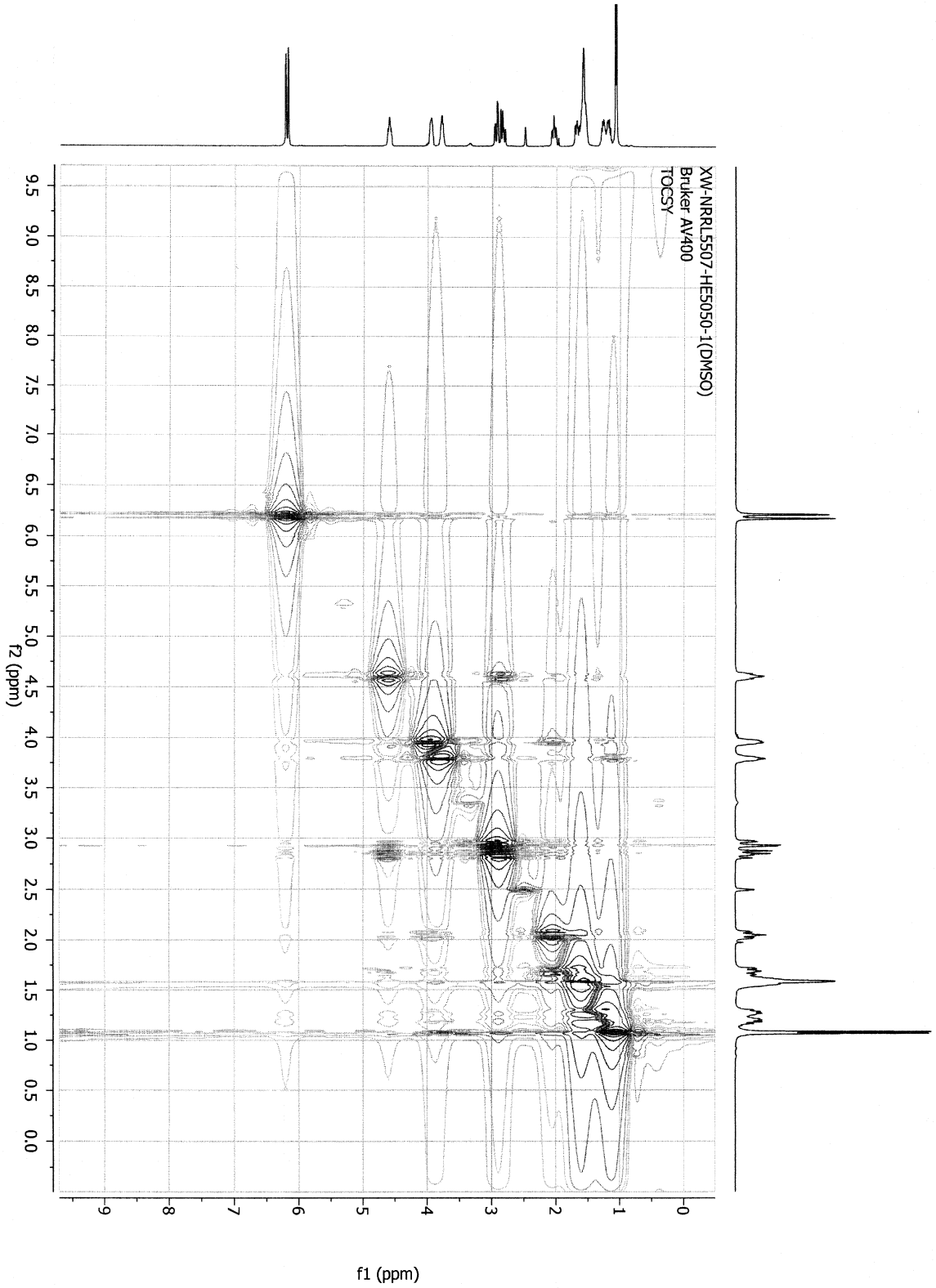
HMBC spectrum of **20**



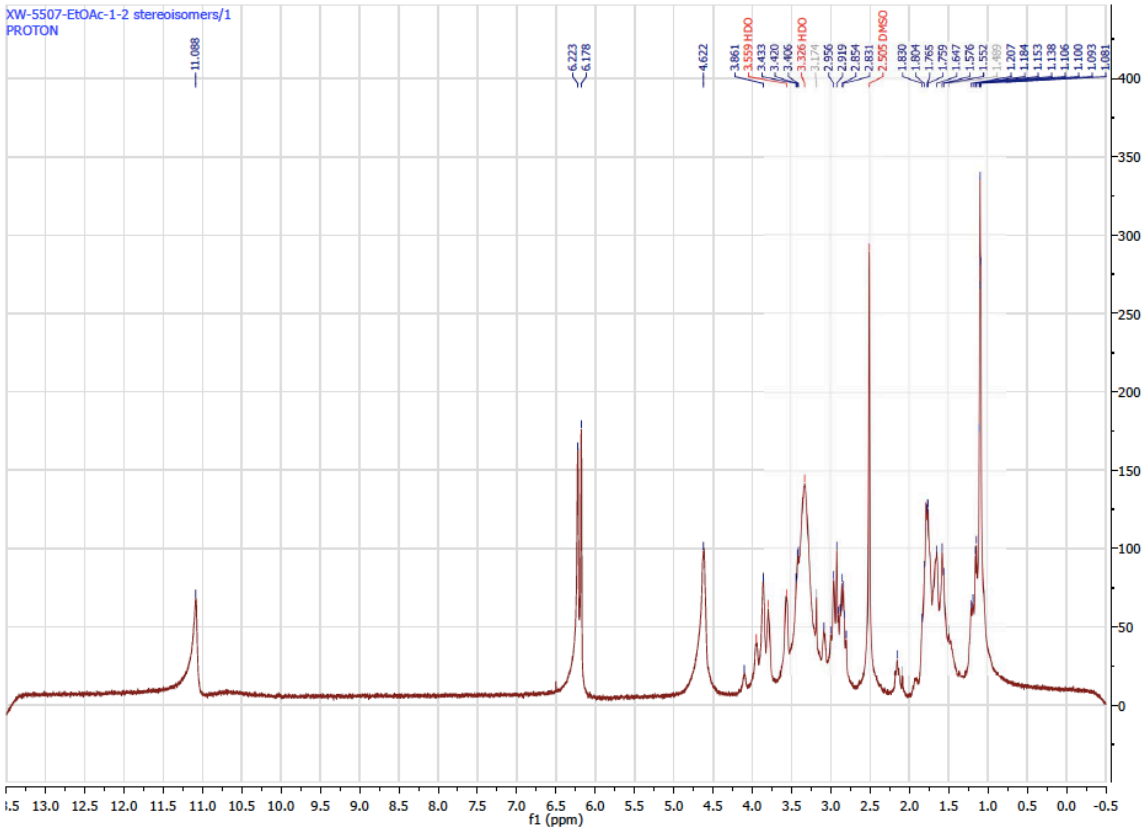
HMQC spectrum of **20**



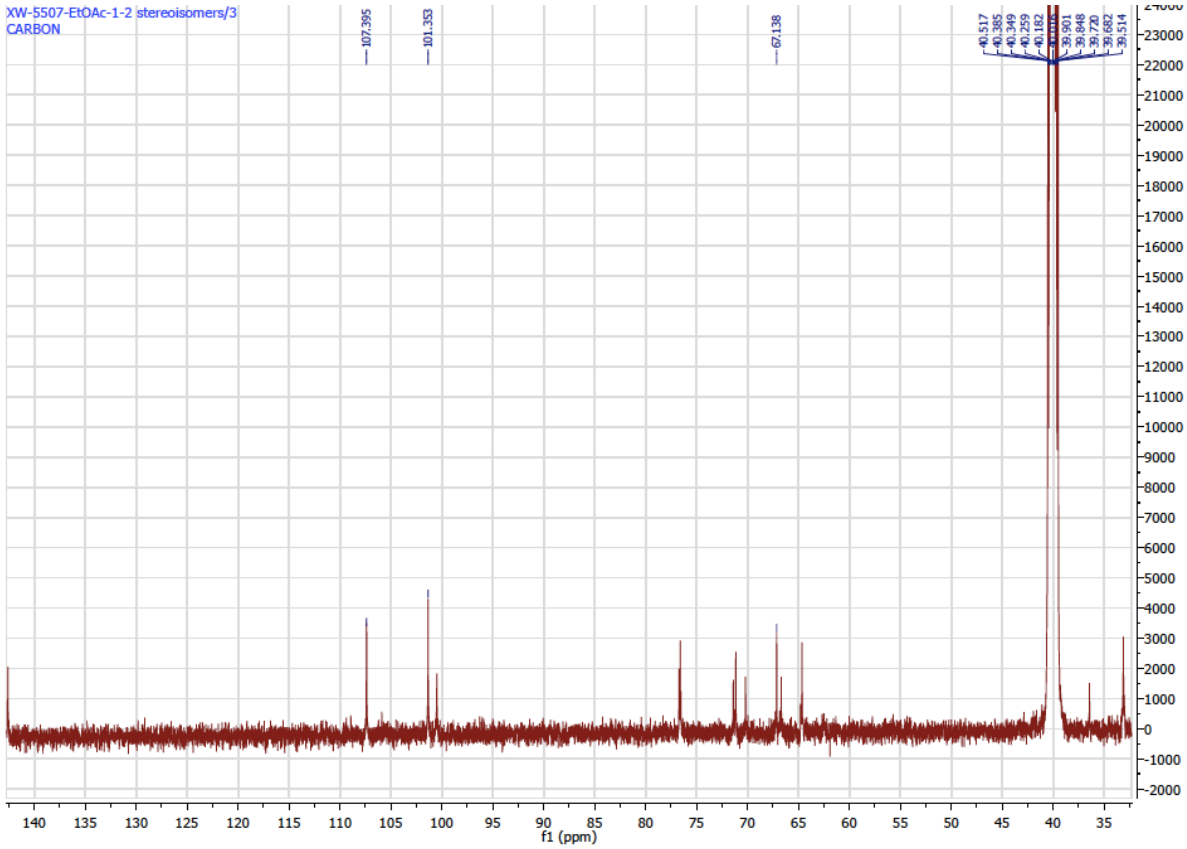
TOCSY spectrum of **20**



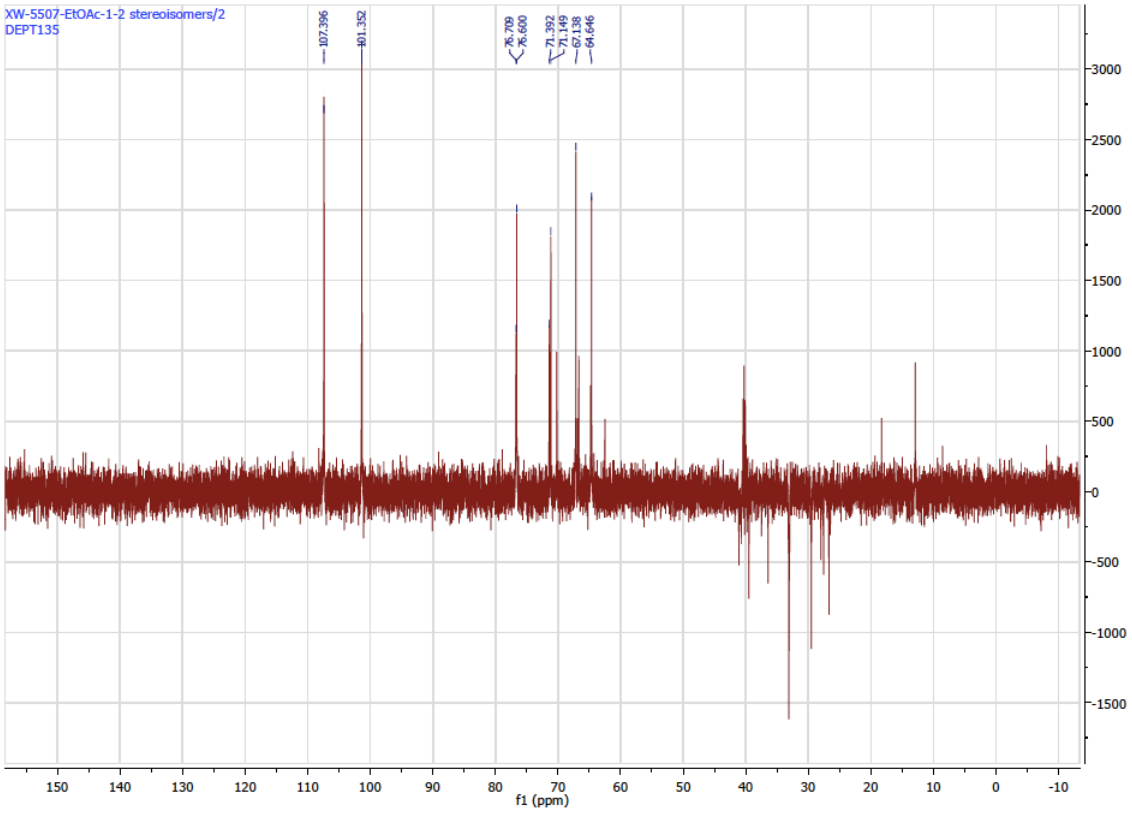
^{1}H NMR of isocladosporin (**21**)



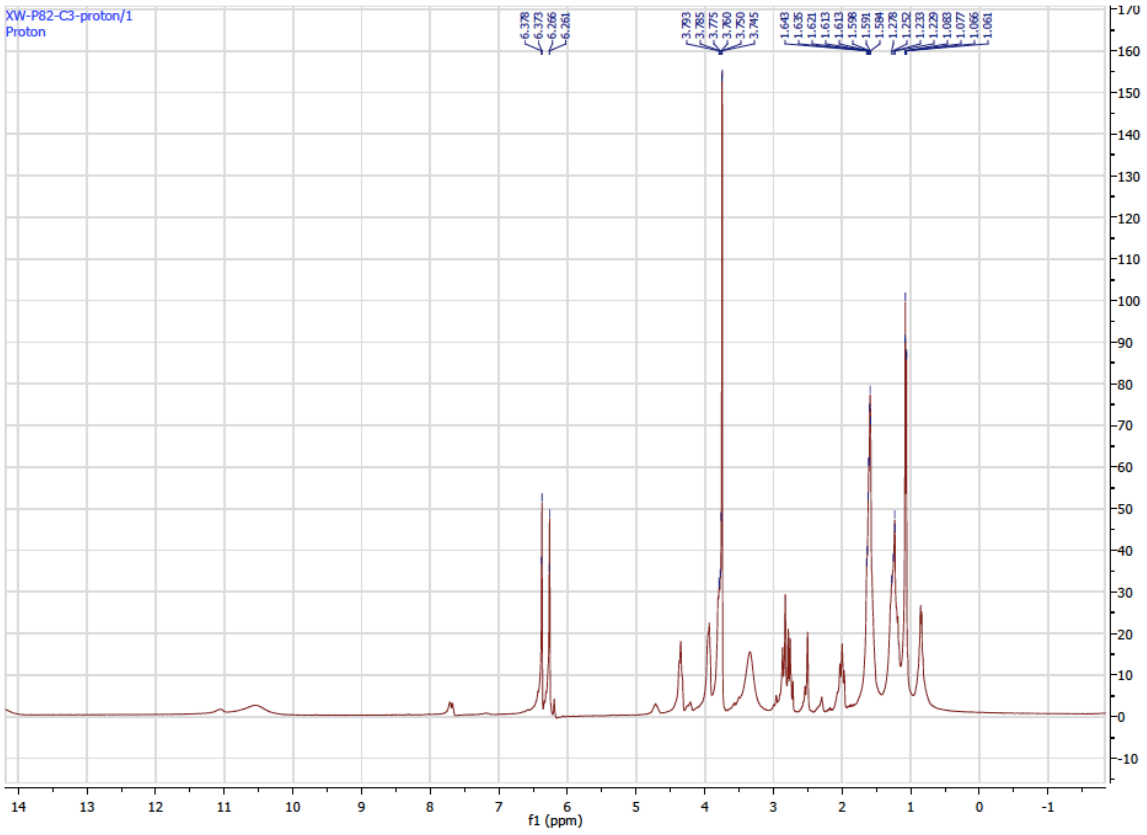
^{13}C NMR of **21**


XW-5507-EtOAc-1-2 stereoisomers/3
CARBON
107.395
101.353
67.138
40.517
40.385
40.349
40.259
40.182
39.901
39.848
39.720
39.682
39.514
f1 (ppm)

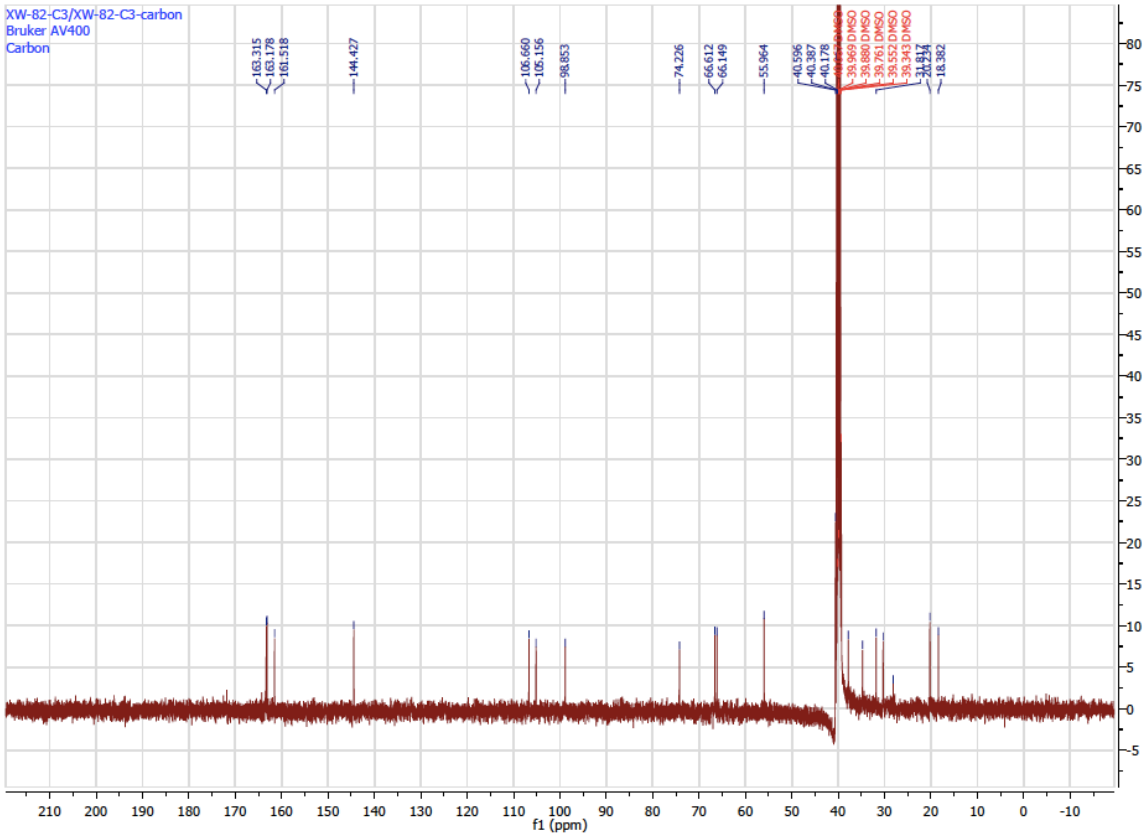
DEPT spectrum of **21**



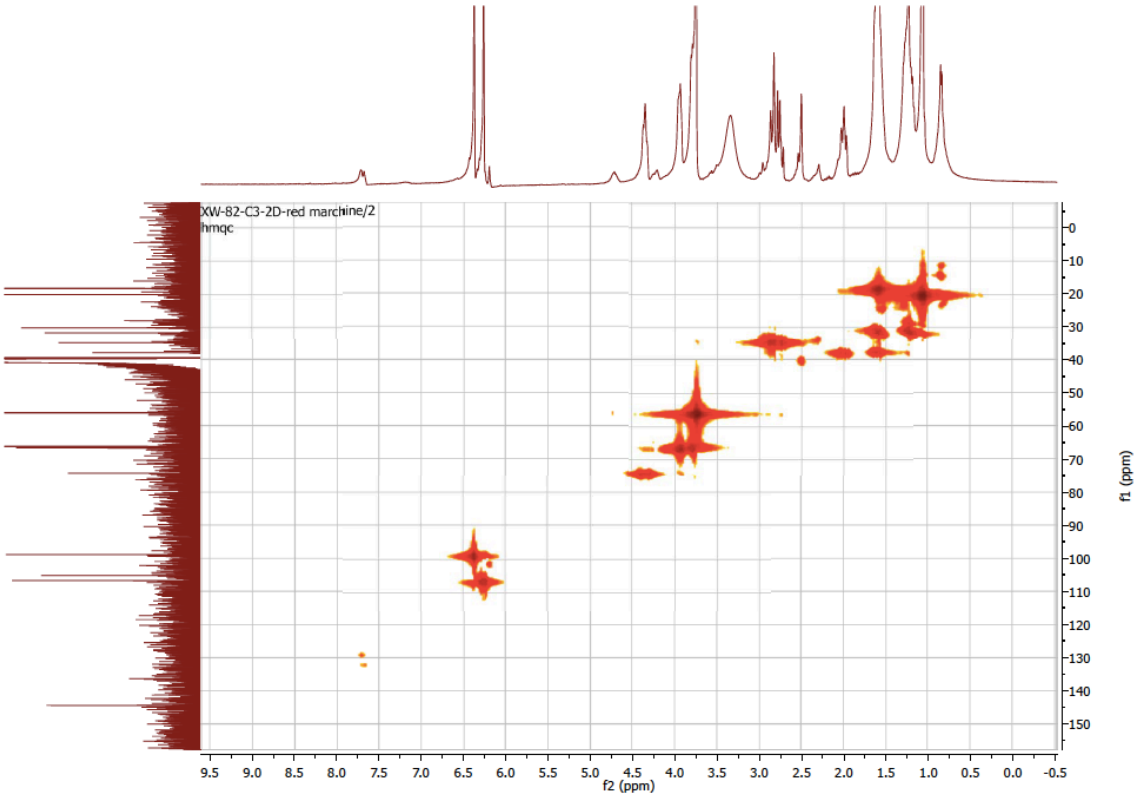
^{1}H NMR spectrum of asperentin-8-methyl ether (**34**)



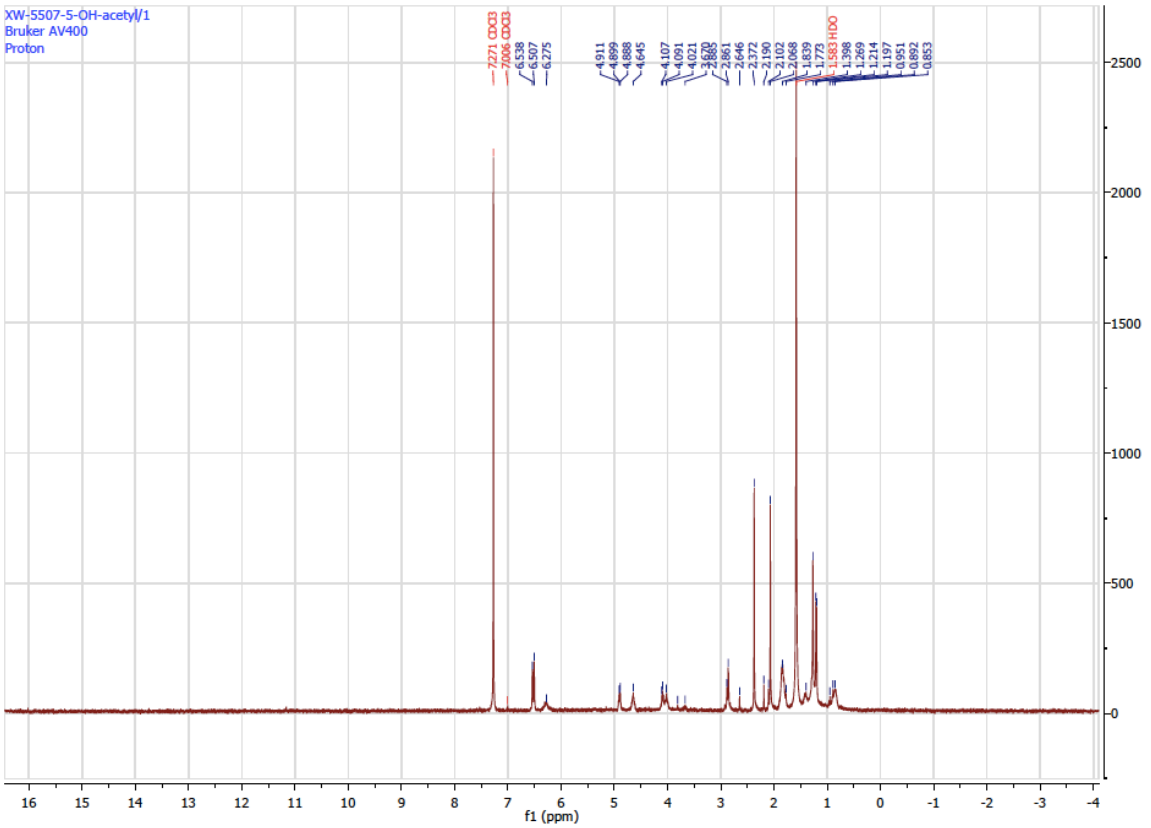
^{13}C NMR of **34**


XW-82-C3/XW-82-C3-carbon
Bruker AV400
Carbon
163.315
163.178
161.518
144.427
106.660
105.156
98.853
74.226
66.612
66.149
55.964
40.596
40.387
40.178
39.969 DMSO
39.880 DMSO
39.761 DMSO
39.552 DMSO
39.343 DMSO
31.817
20.234
18.382
f1 (ppm)

HMQC spectrum of 34



^{1}H NMR of 6, 5'-diacetyl cladosporin (36)



APPENDIX: C

Representative Publications

Antifungal Metabolites from the Roots of *Diospyros virginiana* by Overpressure Layer Chromatography

by **Xiaoning Wang**[a]), **Eman Habib**[a]), **Francisco León**[a]) [b]), **Mohamed M. Radwan**[c]) [d]), **Nurhayat Tabanca**[e]), **Jiangtao Gao**[a]), **David E. Wedge**[*e]), and **Stephen J. Cutler**[*a])[c])

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A preparative overpressure layer chromatography (OPLC) method was successfully used for the separation of two new natural compounds, 4-hydroxy-5,6-dimethoxy-2-naphthaldehyde (**1**) and $\Delta^{12,13}$-20,29-dihydrobetulin (**2**) together with nine known compounds including 7-methyl-juglone (**3**), diospyrin (**4**), isodiospyrin (**5**), shinanolone (**6**), lupeol (**7**), betulin (**8**), betulinic acid (**9**), betulinaldehyde (**10**), and ursolic acid (**11**) from the acetone extract of the roots of *Diospyros virginiana*. Their identification was performed with mono and bidimensional NMR spectroscopy and HR-ESI-MS methods. All the isolated compounds were evaluated for their antifungal activity against *Colletotrichum fragariae*, *C. gloeosporioides*, *C. acutatum*, *Botrytis cinerea*, *Fusarium oxysporum*, *Phomopsis obscurans*, and *P. viticola* using *in vitro* micro-dilution broth assay. The results indicated that compounds **3** and **5** showed high antifungal activity against *P. obscurans* at 30 μM with 97.0 % and 81.4 % growth inhibition and moderate activity against *P. viticola* (54.3 % and 36.6 %). It appears that an optimized OPLC system offers a rapid and efficient method of exploiting bioactive natural products.

Introduction. −Discovery of new crop protections from natural products has received considerable interest as alternatives to synthetic agrochemicals for use as pest and disease control agents. This stems from these generally being safer for human health and the environment. Biologically active natural products can be used in modern crop protections or can serve as lead structures for the development of new semi-synthetic analogs. Our current research efforts are to

identify natural product-based fungicides. In this research program, we evaluated 37 plant extracts using a direct bioautography bioassay to detect antifungal activity against *Colletotrichum*. Of these, *Diospyros virginiana* root extract showed the most promising activity. Based on preliminary screening results, a bioassay-guided fractionation of *D. virginiana* root extract was conducted in order to isolate and identify the pure metabolites possessing antifungal activity. *Diospyros* belongs to the family Ebenaceae that contains approximately 500 species of trees and shrubs [1], of which more than 350 species are distributed in tropical and subtropical regions worldwide [2-3]. The most well-known species is *Diospyros kaki*, which originated in East China and has been cultivated in Japan for centuries [4]. Some of the *Diospyros* species, such as *D. peregrina* and *D. melanoxylon*, have been used in folk medicine for the treatment of inflammation, urinary discharges and enrichment of blood [5]. *Diospyros virginiana*, the American persimmon, is native to North America and the fruits were reported to show cholesterol lowering activity, and usefulness to treat bloody stools, thrush and sore throats [6]. The literature shows reports of nine compounds being isolated and identified from either the leaves or the wood of *D. virginiana* [7-9]. However, there are no reports in the literature on chemical fractions from the roots of *D. virginiana*.

OPLC was developed by Tyihák and Mincsovics in the late seventies [10]. In this process, a TLC plate is covered by a sheet of flexible material and subjected to a high external pressure. The high external pressure, which is generated by a programmable pump, pushes the mobile phase through the analytical or preparative adsorbent layer. This allows a faster separation and more compact spots than conventional TLC. Generally, the R_f of the migrated compounds is two to five times greater than in conventional TLC. OPLC also offers an advantage over traditional TLC as it is a sealed system, which results in less solvent loss through evaporation [11-12]. As

such, OPLC has been widely used in analytical and preparative applications [13-15]. Compared to conventional methods, OPLC offers a more rapid and reliable method for the isolation of naturally occurring compounds. In the present paper, we report the use of a preparative OPLC technique to isolate co-migrating bioactive metabolites from the roots of *Diospyros virginiana*.

Results and Discussion. −The roots of *D. virginiana* were extracted using acetone in a Soxhlet extractor. The constituents of this extract were separated by flash chromatography, OPLC and preparative TLC. A new naphthyl derivative, 4-hydroxy-5,6-dimethoxy-2-naphthaldehyde (**1**), and a triterpene, $\Delta^{12,13}$-20,29-dihydrobetulin (**2**) were isolated from a natural source for the first time, along with nine known compounds: 7-methyl-juglone (**3**) [16], diospyrin (**4**) [17], isodiospyrin (**5**) [16-18], shinanolone (**6**) [19], lupeol (**7**) [20], betulin (**8**) [21], betulinic acid (**9**) [22], betulinaldehyde (**10**) [23], and ursolic acid (**11**) [24]. Although they are common in most of the Ebenaceae family [5], compounds **10** and **11** were isolated for the first time from *D. virginiana*. The structures of the known compounds were confirmed by comparison of their spectroscopic data (MS, ^{1}H and ^{13}C NMR) with literature values.

The HR-ESI-MS of compound **1** indicated a molecular formula $C_{13}H_{12}O_4$ and its IR spectrum exhibited characteristic absorption bands for hydroxyl and conjugated carbonyl groups. The ^{1}H NMR spectrum showed signals for an aldehyde group at δ 10.03 ppm, one aromatic AB system at δ 7.37 (d, J = 9.0 Hz) and 7.78 (d, J = 9.0 Hz). The above data suggested a 2-naphthaldehyde structure with the presence of two methoxyl groups at δ 4.06, and 4.13 ppm, and a hydroxyl substituent at δ 9.75 ppm. The ^{13}C-NMR spectrum showed thirteen signals with ten typical aromatic carbons. Assignment of structure was accomplished by HMBC experiment. The

NOESY experiment showed a correlation between the methoxyl group at δ 4.06 and H-7 (δ_H 7.37, d) and methoxyl group at δ 4.13 and the hydroxyl signal at δ 9.75 ppm, corroborating the location of the methoxyl groups at C(5) and C(6). Thus, **1** was characterized as 4-hydroxy-5,6-dimethoxy-2-naphthaldehyde.

Compound **2** was obtained as a white solid. The molecular formula was determined as $C_{30}H_{51}O_2$ by HR-ESI-MS ($[M + H]^+$ at m/z = 443.3874). The ^{13}C NMR data of **2** indicated the presence of 30 carbons comprising seven methyl groups, ten methylenes, seven methines and six quaternary carbons. The seven methyl groups resonated at δ_H 0.79 (s, 6H), 0.93 (d, 6H, J = 6.8), 0.98 (s, 3H), 0.99 (s, 3H), and 1.10 (s, 3H) in the ^1H NMR spectrum indicating the lupane triterpene skeleton for **2** [25]. The ^{13}C NMR spectrum also revealed the presence of one olifenic methine (δ(C) 125.0) correlated in the HMQC spectrum with the proton at δ(H) 5.20 (H-C(12)). The last proton showed HMBC correlation with C(9), C(14), and C(18), this correlation led to the placement of the double bond between C(12) and C(13). This was confirmed by the COSY correlation between H-C(12) (δ(H) 5.20) and H_2-C(11) (δ(H) 1.90) and further supported by the HMBC correlation of H_3-C(27) (δ(H) 1.10) and C(13) (δ(C) 136.7) (*Fig. 1*). From these data, the compound **2** was deduced to be $\Delta^{12,13}$-20,29-dihydrobetulin. This is the first report for the full spectral data and isolation of **2** from a natural source, although it has been prepared synthetically and only the ^1H NMR data was reported [26].

The antifungal activity of compounds **1-11** was examined using a 96-well micro-dilution broth assay against the plant pathogens: *Botrytis cinerea*, *Fusarium oxysporum*, *Phomopsis obscurans*, *P. viticola* and three *Colletotrichum* species. *Phomopsis* species were the most

sensitive fungi to these compounds. 7-methyl-juglone (**3**) and isodiospyrin (**5**) showed the most antifungal activity against *P. obscurans*. Compound **3** showed 97.0 % growth inhibition of *P. obscurans* at 120 h at 30 µM, whereas compound **5** showed 81.4 % growth inhibition. The antifungal activity of **3** and **5** at 30 µM against *P. viticola* was 53.4% and 57.7%, respectively.

The newly reported compounds, 4-Hydroxy-5, 6-dimethoxy-2-naphthaldehyde **(1)** and $\Delta^{12,13}$-20,29-dihydrobetulin (**2**) at 30 µM showed weak antifungal activity with 26.9 % and 22.1 %, respectively (*Fig. 2*). Compound **3** and compound **5** at 30 µM caused 54.3 % and 36.6 % growth inhibition of *P. viticola* at 120 h. Any test compound possessing <50% growth inhibition at 30 µM is considered to have weak antifungal activity in this bioassay. Compound **1** was more active against *P. vitricola* at 120 h than at 144 h. This response is often seen when an inducible enzyme system is turned on and a compound is detoxified by the fungus. However, the upward slope of the graph for compound **1** in *Fig 3* from the low to high concentration is indicative of precipitation in the aqueous microdilution broth assay. Because the microtiter plate reader measures changes in optical density it does not discriminate between fungal growth and precipitation. Compound 1 appears to have come out of solution at the higher concentration (30 µM) in the *P. viticola* testing (*Fig 3*). Lipophilic compounds are problematic in *in vitro* aqueous bioassays and follow-up antifungal testing will take place using a detached leaf bioassay [27]. Interpretation of the graphical results indicate that compounds **2**, **3**, and **5** appear to remain soluble at 120 h and 144 h.

Commercial fungicides standards captan and benomyl are significantly more active than any of the compounds tested. Azoxystrobin, which is commercially used to control *Colletotrichum* and *Botrytis* diseases, shows poor activity against *P. viticola*. Both captan and

azoxystrobin show 100% growth inhibition at 3 μM against *P. obscurans*. Although once an excellent agent for controlling anthracnose and other diseases of strawberries and ornamentals, resistance developed by pathogens has resulted in benomyl being less useful [28]. While the test compounds appear inherently antifungal, we hypothesize that these compounds are probably present in the plant as constitutive defense compounds that act to deter infection or fungal growth. Since these compounds were found without elicitation they are probably constitutive in nature [29-30] and may have a potential role in preventing fungal infection in *D. virginiana*.

Conclusions. − Using a bioassay-guided fractionation of the acetone extract *D. virginiana* were able to isolate the antifungal constituents of the roots of this plant. This research demonstrates that OPLC is a powerful technique that can be used to separate and isolate co-migrating natural products produced by plants. These constituents were tested for the first time against *Colletotrichum* spp, *B. cinerea*, *F. oxysporum*, *P. obscurans*, and *P. viticola*. The most promising agricultural lead compounds against the pathogen *P. obscurans* are **3** and **5**. Phomopsis leaf blight and fruit rot is a serious disease with strawberries and causes serious economic loss of this fruit each year. Our results suggest that compounds **3** and **5** warrant further *in vivo* testing as plant protectants to control *Phomopsis* species [27].

Acknowledgments. −The project described was supported by Grant Number 5P20RR021929 from the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Center for Research Resources

or the National Institutes of Health. This investigation was conducted in a facility constructed with support from research facilities improvement program C06 RR-14503-01 from the NIH National Center for Research Resources. Francisco León was supported by JAE-Postdoctoral Program from the Ministerio de Ciencia e Innovación, Spain. The authors thank J. Linda Robertson and Ramona Pace for assistance in performing various bioassays and J'Lynn Howell for the photographic expertise information.

Experimental Part

Chemistry

General. UV spectra were obtained in MeOH using a Varian Cary 50 spectrophotometer and IR spectra were recorded using Bruker Tensor 27 spectrophotometer. ^{1}H- and ^{13}C-NMR spectra were obtained on Bruker model AMX-500 and 400 NMR spectrometers with standard pulse sequences, operating at 500 and 400 MHz for ^{1}H and 125 and 100 MHz for ^{13}C. High resolution electrospray ionization mass spectroscopy (HR-ESI-MS) was recorded on a Micromass Q-Tof Micro mass spectrometer with a lock spray source. OPLC separations were performed with the Personal OPLC 50 instrument (OPLC-NIT, Budapest, Hungary) at 50 bars external pressure. OPLC silica gel layer, F_{254} 20 × 20 cm on glass plate (LG 011, OPLC-NIT Ltd, Budapest Hungary) with 200 μm sorbent thickness, 11 μm particle size, 6 nm pore size, and 20 × 20 cm on aluminum sheet (BSLA 001, OPLC-NIT Ltd, Budapest Hungary) with 200 μm sorbent thickness, 5 μm particle size, 6 nm pore size. An AS-30 sample applicator (DESAGA, Wiesloch, Germany) was used for the sample application. Classical TLC analysis was performed on silica gel 60 F_{254} 20 × 20 cm on aluminum sheet (Gibbstown, New Jersey, USA). Detection was carried out under UV light (254 nm, 366 nm) and visualization made with vanillin-H_2SO_4 (1 g

vanillin in 100 ml of 20 % H_2SO_4 in EtOH) reagent followed by heating at 105 ºC for 5 min. ACS-grade solvents, acetonitrile, acetone, chloroform, dichloromethane (DCM), diethyl ether, ethyl acetate (EtOAc), EtOH, *n*-hexane, isopropanol (IPA), methanol and toluene from Fisher Scientific (New Jersey, USA) were used for silica gel column chromatography and TLC separations. HPLC-grade solvents (acetonitrile, chloroform and DCM) from Sigma-Aldrich (St. Louis, USA) were used for OPLC chromatograms.

Plant material. The roots of *D. virginiana* were collected from the Missouri Botanical Garden, U.S.A., in June 2009. The plant material was identified by Dr. Vaishali C. Joshi, and a voucher specimen CON310700-2-A was deposited at the National Center for Natural Products Research, School of Pharmacy, University of Mississippi.

Extraction and Isolation. Dried powdered roots (66 g) were extracted in a Soxhlet extractor with acetone (600 ml) for 8 h and the extract was subsequently evaporated under vacuum to yield the dry residue (2 g). The crude extract was fractionated on a silica gel column (100 g, 50 × 5 cm, ChemGlass) using *n*-hexane (500 ml), 5 % EtOAc in *n*-hexane (600 ml), chloroform (600 ml), 5 % IPA in chloroform (600 ml) and methanol (600 ml), respectively. Four fractions were obtained in total F-1 to F-4. Direct-bioautography guided assay showed that the activity was found to reside in F-1 (120 mg) and F-2 (240 mg), with some activity in F-3 (710 mg), while F-4 (800 mg) did not show activity against three notorious plant pathogenic fungi of the *Colletotrichum* species. A portion of F-1 (10 mg) showed one major product and was purified using preparative TLC (SiO_2, *n*-hexane-chloroform 1:1) to furnish 7-methyl juglone (**3**, 1.5 mg). A portion of F-2 (20 mg) was purified using OPLC with DCM as the eluent. Elution conditions were as follows:

flash volume, 300 µl; eluent volume, 30,000 µl; flow-rate, 500 µl min^{-1}; development time, 3606 s; external pressure, 50 bars. Eighty-eight sub-fractions (1 ml / fraction) were obtained in total, of which sub-fractions 49-54 gave betulinaldehyde (**10**, 0.8 mg). Sub-fractions 72-88 afforded ursolic acid (**11**, 1.3 mg). Sub-fractions 28-37 were combined (7.9 mg) and further purification was performed by OPLC with *n*-hexane-ether (6/4, v/v, 20,000 µl; flash volume, 300 µl; flow-rate, 500 µl min^{-1}; development time, 2406 s) as eluent and 66 sub-fractions (1 ml / fraction) were obtained, among which sub-fractions 10'-18' furnished lupeol (**7**, 2 mg). Sub-fractions 19'-66' (marked as fraction A) were combined to give a mixture of 3.6 mg containing four compounds with close R_f values in several TLC systems (SiO_2, *n*-hexane-EtOAc 6:4, DCM-IPA 9:1, $CHCl_3$-MeOH 19:1). In order to obtain enough material for further purification, OPLC procedures were repeated six times for F-2 using the same conditions, then fraction A (19.6 mg) was obtained and purified by OPLC with DCM (100 %, 20,000 µl; flash volume, 300 µl; flow-rate, 400 µl min^{-1}; development time, 3007 s) as mobile phase. Sub-fraction 12″ yielded a new product 4-hydroxy-5, 6-dimethoxy-2-naphthaldehyde (**1**, 1 mg). Sub-fractions 9″-11″ gave diospyrin (**4**, 2 mg). Sub-fractions 14″-21″ afforded isodiospyrin (**5**, 2 mg). Fraction 3 (24 mg) was chromatographed using OPLC with $CHCl_3$-EtOAc (95/5, v/v, 40,000 µl) with flash volume 300 µl, flow rate 500 µl min^{-1} and an elution time of 4806 s. One hundred and twenty-three sub-fractions (1 ml / fraction) were collected. Sub-fractions 54-66 yielded betulin (**8**, 3.4 mg). Sub-fractions 67-90 (7.4 mg), containing at least four compounds, were purified by OPLC with $CHCl_3$-acetonitrile (98/2, v/v) and flash volume 300 µl, flow rate 300 µl min^{-1} and a total elution time of 4806 s. Sub-fractions 79'-87' gave shinanolone (**6**, 1.5 mg). The other minor compounds were in such small quantity that they were impossible to isolate. In order to obtain enough material for further purification, flash chromatography of F-3 (400 mg) using a BIOTAGE

(Isolera One) with $CHCl_3$-EtOAc (0-5 %, 200 ml) and $CHCl_3$ (100 %, 201 ml-640 ml), and flow rate, 5 ml min^{-1} (Si, SNAP 25 g column) was conducted. A total of 81 sub-fractions (1 ml/fraction) were collected. Subfractions 38-51 were combined to yielded a 30 mg mixture. This mixture was subjected to the OPLC system with $CHCl_3$-acetonitrile (98/2, v/v, 40,000 µl) with flash volume 300 µl, flow rate 250 µl/min and total elution time 9624 s. One hundred and eighty-three subfractions (1 ml / fraction) were obtained. Subfractions 100-112 were combined and identified as betulin (**8**, 2 mg). Subfractions 136-144 yielded betulinic acid (**9**, 1 mg). Subfraction 114-126 gave a new triterpene, $\Delta^{12,13}$-20,29-dihydrobetulin (**2**, 1 mg) as a natural product.

4-hydroxy-5,6-dimethoxy-2-naphthaldehyde (**1**): Yellow amorphous solid; UV (MeOH): λ_{max} nm (log ε): 375 (4.1), 320 (4.2), 270 (3.8); IR (neat): ν_{max} 3332, 2923, 2851, 2360, 2341, 1687, 1372, 1274, 1055 cm^{-1}; ^{1}H- NMR (500 MHz, $CDCl_3$): δ 4.06 (3H, s, OMe), 4.13 (3H, s, OMe), 7.29 (1H, s br, H-C(3)), 7.37 (1H, d, *J*= 9.0 Hz, H-C(7)), 7.78 (1H, d, *J*=9.0 Hz, H-C(8)), 7.81 (1H, s br, H-C(1)), 9.75 (1H, OH-C(4)), 10.03 (1H, s, H-C(11)); ^{13}C-NMR (125 MHz, $CDCl_3$): δ 56.67 (OMe), 62.20 (OMe), 106.14 (C(3)), 115.46 (C(7)), 121.49 (C(10)), 126.02 (C(1)), 127.19 (C(8)), 130.120 (C(9)), 134.09 (C(2)), 143.46 (C(5)), 149.95 (C(6)), 154.28 (C(4)), 191.79 (C(11)); HR-ESI-MS: *m*/*z* = 233.0812 ($[M + H]^+$, $C_{13}H_{13}O_4$; calc. 233.0814).

$\Delta^{12,13}$-20,29-dihydrobetulin (**2**): White amorphous solid; $[\alpha]^{20}_D$ = +79.2 (*c* 0.05, MeOH); IR (KBr): ν_{max} 3340, 2940, 2867, 1446, 1372, 1027 cm^{-1}; ^{1}H-NMR (400 MHz, $CDCl_3$): δ 0.73 (1H, d, *J*=7.2 H-C(5)), 0.79 (6H, s, Me-24, Me-26), 0.89 (1H, m, , H-C(20)), 0.93 (6H, d, *J*= 6.8 Hz, Me-29, Me-30), 0.98 (3H, s, *J*=9.0 Hz, Me-25), 0.99 (3H, s, Me-23), 1.10 (3H, s, Me-27), 1.20-1.81 (8CH_2), 1.90 (2H, m, H_2-C(11)), 3.19 (1H, d, *J*=10.4, H-C(28^a)), 3.22 (1H, m, H-C(3)), 3.53 (1H, d, *J*=10.4, H-C(28b)), 5.13 (1H, bs, H-C(12)); ^{13}C NMR (100 MHz, $CDCl_3$): δ 15.6 (C(29)), 15.7 (C(24)), 16.7 (C(25)), 17.3 (C(26)), 18.3 (C(6)), 21.3 (C(30)), 23.2 (C(21)), 23.3 (C(27)),

23.4 (C(11)), 25.9 (C(2)), 27.2 (C(15)), 28.1 (C(23)), 30.6 (C(16)), 32.8 (C(7)), 35.2 (C(1)), 36.9 (C(14)), 38.7 (C(22)), 38.0 (C(4)), 39.3 (C(20)), 39.4 (C(19)), 40.0 (C(10)), 42.0 (C(8)), 42.3 (C(17)), 47.6 (C(18)), 54.0 (C(9)), 55.1 (C(5)), 69.9 (C(28)), 79.0 (C(3)), 125.0 (C(12)), 136.7 (C(13)). HR-ESI-MS *m/z* = 443.3874 ($[M + H]^+$, $C_{30}H_{51}O_2$; calc. 443.3889).

Biological Assay

Direct-bioautography assay. Bioautography procedures were described in our previous studies [31-32]. The acetone extract of *D. virginiana* roots was applied at 80 and 160 µg/spot in chloroform onto a silica plate. Technical fungicide grade standards benomyl, cyprodinil, azoxystrobin, and captan (Chem Service Inc., West Chester, PA) were used as positive controls at 2 mM in 2 µl of 95% ethanol. TLC profiles of F-1 to F-4 in chloroform were tested against *Colletotrichum* spp and mild polar compounds appear to be responsible for antifungal activity.

Micro-dilution broth assay. A standardized 96-well micro-dilution broth assay developed by Wedge and Kuhajek [33] was used to evaluate the antifungal activity of pure compounds from *D. virginiana* that were identified as active by bioautography.

Isolates of *Colletotrichum acutatum* Simmonds, *Colletotrichum fragariae* Brooks, *Colletotrichum gloeosporioides* (Penz.) Penz & Sacc. In Penz, *Botrytis cinerea* Pers.:Fr, *Fusarium oxysporum* Schlechtend:Fr, *Phomopsis obscurans* (Ellis and Everh.) *B. sutton*, and *P. viticola* Sacc., were used to evaluate the antifungal activity of the test compounds using *in vitro* micro-dilution broth assay. Each fungus was challenged in a dose-response format using test compounds where the final treatment concentrations were 0.3, 3.0 and 30.0 µM. Technical grade commercial fungicides captan, azoxystrobin, and benomyl, which represent three different modes of actions, were used as positive fungicide standards. Each compound was evaluated in

duplicate and the experiment was performed three times in time. Mean absorbance and standard errors were used to evaluate fungal growth after 48 and 72 h, except for *P. obscurans* and *P. viticola* (120 and 144 h).

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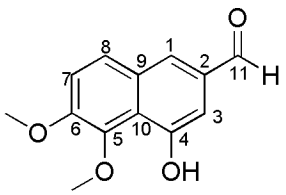
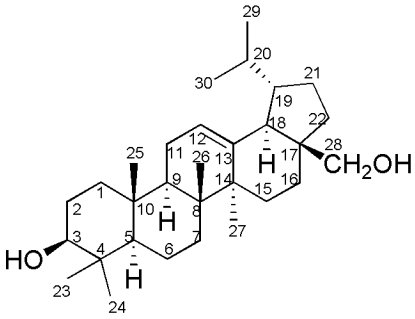
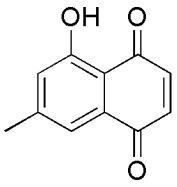


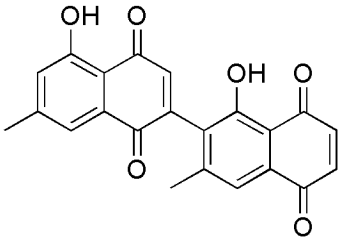
4-hydroxy-5,6-dimethoxy-2-naphthaldehyde (**1**)



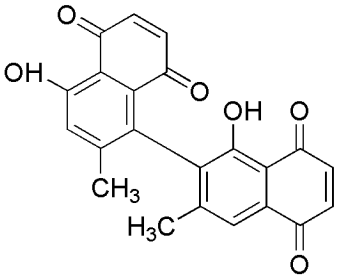
$\Delta^{12,13}$-20,29-dihydrobetulin (**2**)



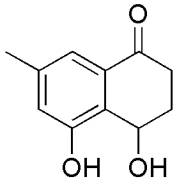
7-methyl-juglone (**3**)



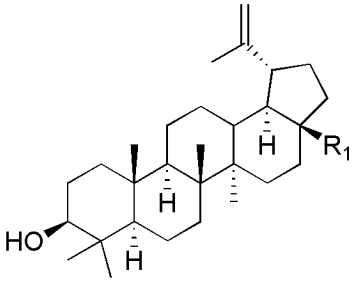
diospyrin (**4**)



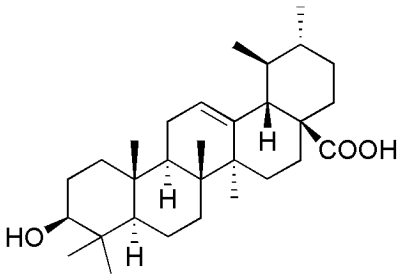
isodiospyrin (**5**)



shinanolone (**6**)



lupeol (**7**) $R_1=CH_3$
betulin (**8**) $R_1=CH_2OH$
betulinic acid (**9**) $R_1=COOH$
betulinaldehyde (**10**) $R_1=CHO$



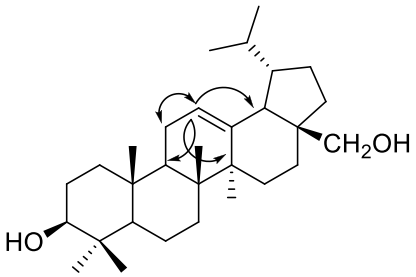
ursolic acid (**11**)

Figures Legends

Fig. 1. *Relevant ^{1}H-^{13}C HMBC (→) and ^{1}H-^{1}H COSY (↔) correlations of* ***2***

Fig. 2. Mean fungal growth inhibition (%) of *Phomopsis obscurans* after exposure to **1**, **2**, **3** and **5** using a dose-response format at 120 and 144 h. Abreviations: Cap: Captan; Azo: Azoxystrobin; Ben: Benomyl

Fig. 3. Mean fungal growth inhibition (%) of *Phomopsis viticola* after exposure to **1**, **2**, **3** and **5** using a dose-response format at 120 and 144 h. Abreviations: Cap: Captan; Azo: Azoxystrobin; Ben: Benomyl



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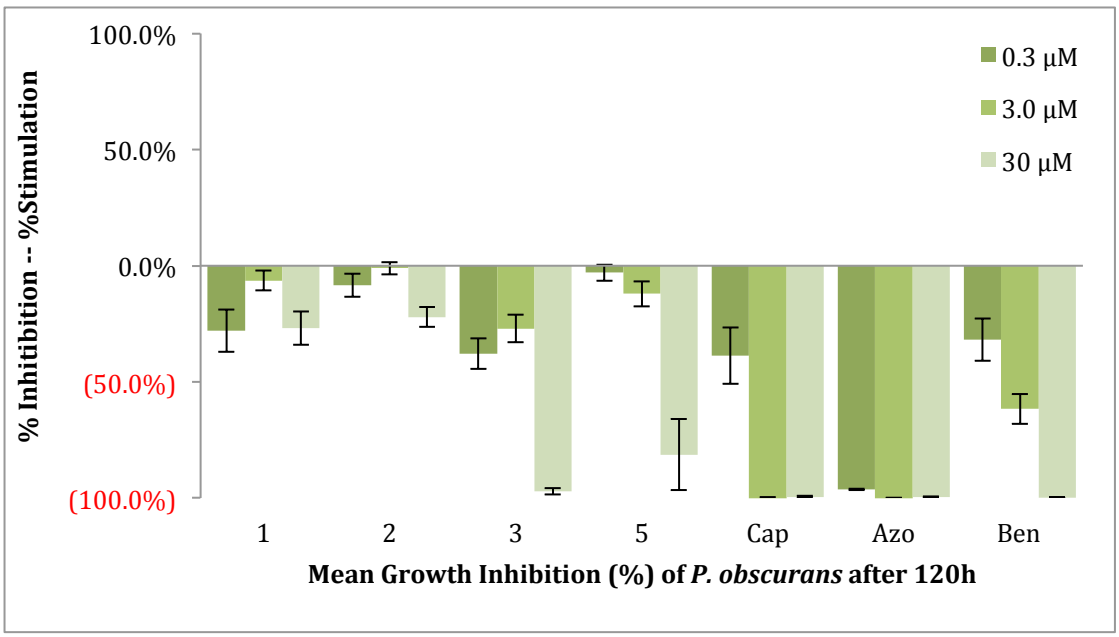

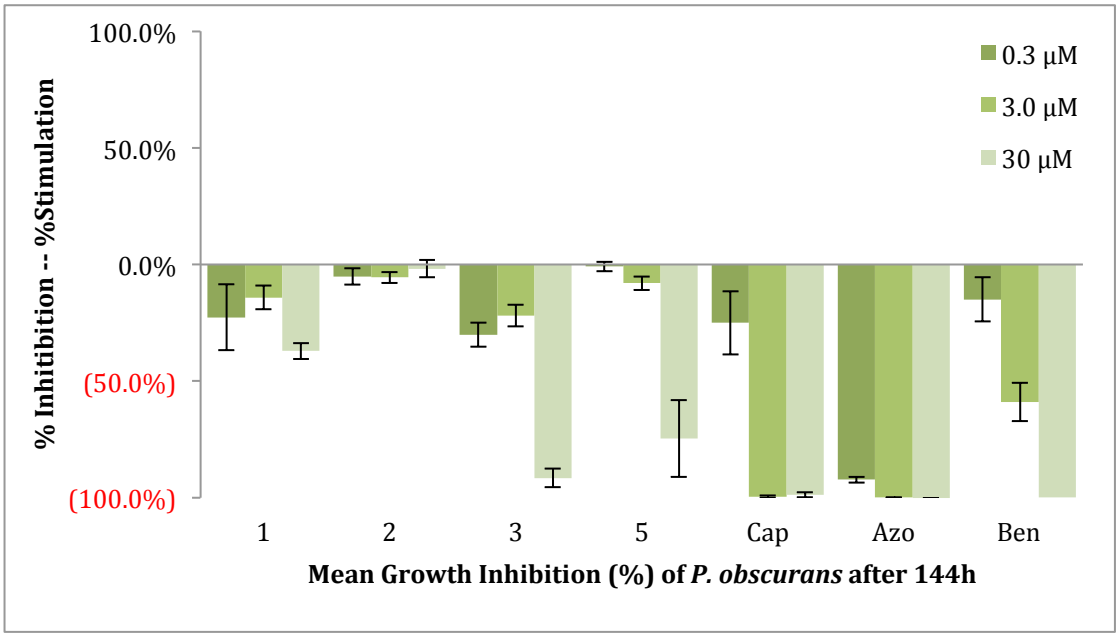

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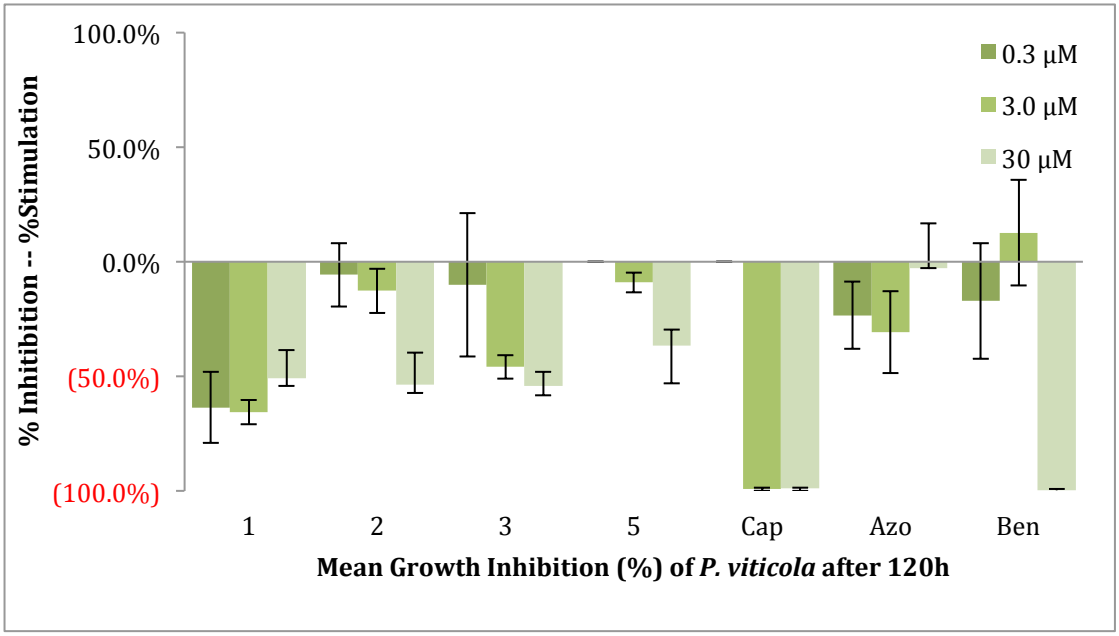

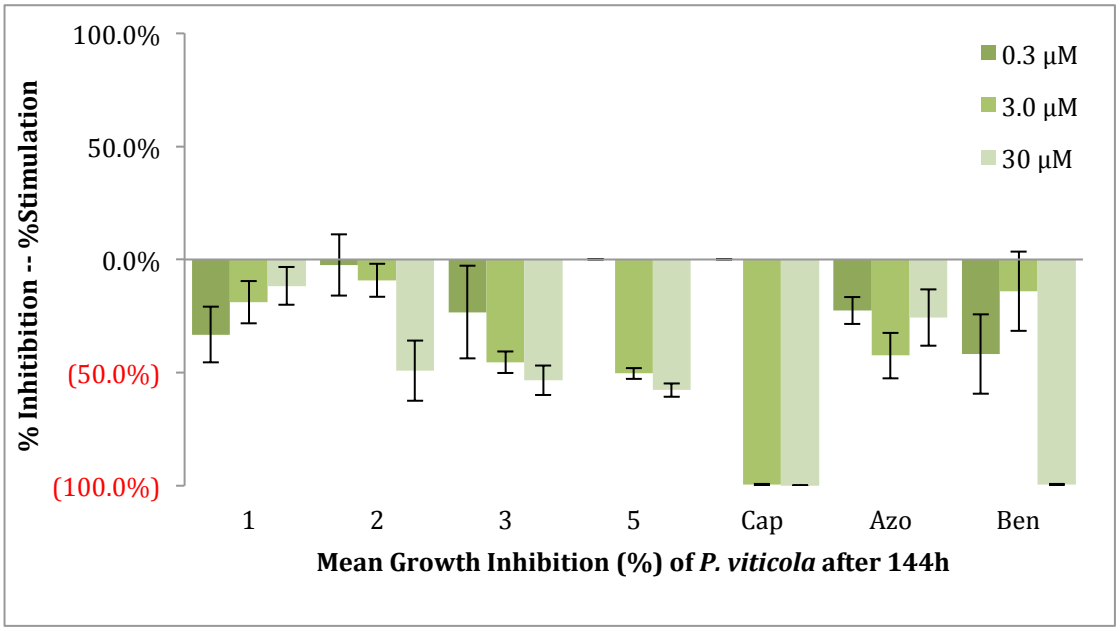

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Development of A Minaturized 24-well Strawberry Leaf Disk Bioassay for Evaluating Natural Fungicides

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Abstract

Introduction

Fruit rot diseases of strawberry (*Fragaria x ananassa* Duch.) are serious problems for strawberry producers in many areas of the world [1](Maas, 1998) and are particularly severe in the southeastern United States where diseases are often enhanced by warm temperatures and frequent rains during the harvest season. Losses by Florida strawberry growers due to disease were estimated to be 10 to 15% of the crop whose farm gate revenue averages over $100 million each season [2] (Legard, et al., 1997). Anthracnose diseases caused by *Colletotrichum* spp. [3,4,5] (Smith, 1998a, 1998b, 1998c) can be especially devastating since other plant parts may be infected in addition to the fruit.

Effective disease control strategies for crop plant pathogens often rely on the use of fungicides. Wedge et al. [6], reported on new fungicide management strategies for control of strawberry fruit rot diseases in the Gulf state region and the need for efficacious fungicides to provide growers with more options for controlling disease. Disadvantages of the use of these chemical controls include the development of resistant fungal strains as well as potential environmental and mammalian toxicities. Resistance of many plant pathogens to commonly used commercial fungicides is becoming a serious problem and has limited the number of effective disease control agents. Numerous isolates of *Colletotrichum* spp. developed benomyl insensitivity after several years of use to protect strawberry and other fruit crops [7,8] (Peres et al., 2002, 2004; [9,10]Smith and Black, 1993 a-b). *Colletotrichum* species often cause typical symptoms of anthracnose, a disease characterized by sunken necrotic lesions usually bounded by a red margin [11,12] (Freeman et al., 1998; Wedge et al., 1999). Anthracnose diseases of strawberry (*Fragaria* × *ananassa* Duch) are serious problems for fruit and plant production in many areas of the world [14] (Maas, 1998). The pathogens, *Colletotrichum fragariae* A. N. Brooks*, C. gloeosporioides* (Penz.) Penz*.,* and *C. fragariae* CF -75, can occur singly or in combination, and can infect flowers, fruit, leaves, petioles, stolons, and crowns (Howard et al., 1992; [15,3,4,5] Smith 1998 a, b, c).

Fungicide discovery efforts at the USDA–ARS, Natural Products Utilization Research Unit, National Center for Natural Products Research, University, MS, are focused on natural products derived from plants and marine organisms with emphasis on compounds with fungicidal activity [16] (Wedge and Kuhajek, 1998). Along with discovery of new chemistry and our interest in antimicrobial agents from higher plants [17] (Zhou and Wedge, 2007), we have also had to developed novel miniaturized bioassays for evaluating small amounts of

available compound [18,19] (Wedge and Smith, 2006; Abril et al., 2008). Natural antimicrobial agents from plants are generally broad-spectrum compounds with low mammalian and environmental toxicity [17] (Zhou and Wedge, 2007).

Development new plant protectants with a lipophilic nature are limited by the lack of suitable bioassays to properly evaluate these compounds. While the lipophilic nature of phytochemicals and marine compounds allows them to embed themselves into the leaf waxes and persist through rainfall, hence making them potentially useful agrochemicals, this characteristic also makes them very difficult to evaluate in many bioassays. Essential oils and many marine extracts are hydrophobic and insoluble in most aqueous-based antimicrobial disc diffusion and micro-dilution broth bioassays. Direct bioautography on silica gel is our preferred primary screening bioassay and often the best assay for evaluating lipophilic compounds as fungicides for agricultural use because this assay more closely mimics a leaf surface. Classical leaf bioassays used to test fungicide efficacy at concentrations between 625 and 2500 ppm may use 75-100 mg of an experimental compound that is often difficult to obtain. Therefore the need for a novel miniaturized leaf disk bioassay that utilizes small sample sizes, has high throughput capability, and replicates the real leaf surface is of utmost importance to natural product fungicide discovery. This paper presents information about our progress in developing of a new 24-well detached leaf bioassay and we discuss problems associated with evaluating natural product fungicides.

Experimental

Plant preparations

Anthracnose-susceptible 'Chandler' plants were obtained in October 2007 from Shingleton Farms (Statonsburg, NC, USA), planted into 14 cm plastic azalea pots containing Metromix 350 (BWI- Memphis, TN), and placed in the greenhouse without shade. Plants were watered daily and weekly with Peter's 20-20-20 nutritional solution (BWI- Memphis, TN). Plants with any signs of anthracnose or wilt disease were rouged and removed from the greenhouse daily.

24-well Leaf Disk Assay

Three commercial in vitro salt solutions were evaluated for their ability to maintain leaf disks during the in vitro experiment. Half strength Hoagland's No. 2 (H, Phytotechnology Laboratories, Shawnee Mission, KS), 2-N-morpholino ethanesulfonic acid (MES, Sigma M8250, Sigma-Aldrich, St. Louis, MO), Murashige and Skoog basal salt mixture (MS, Sigma M5524), and water (control) were evaluated for their ability to sustain excised leaf discs for 7-10 days. Stock solutions containing 1% Phytogel (Sigma P8169) were prepared as follows: Hoagland's No. 2 solution (0.815 g/L), MES (195.2 mg/L) containing 5 mL Gamborg's Vitamins (Thiamine-HCl 5 mg/L, Pyridoxine-HCl 0.5 mg/L, Nicotinic acid 0.5 mg/L, Myo-inositol 50mg/L), and MS (2.2 g/L). All support solutions were adjusted to 6.5 pH using 1 N NaOH and 0.1 N NaOH. Excised leaf discs were placed onto the 1500 µL of either respective solidified salts/medium contained in each well of the 24-well plate. Plates were maintained in the sterile biological hood overnight prior to pathogen inoculation to allow time for the leaf disks to initiate wound healing and acclimate to the in vitro system. 24-well were plates were sealed with paper-based tape and placed in the incubator under (55±5 $\mu mols \cdot m^{-2} \cdot sec^{-1}$) Ushio Ultra 8 fluorescent lights (UFL-F17T8/741, Interlectric Corp., Warren, PA) with a 12 h photoperiod in a growth chamber at 25º

C for one week. All samples were done in duplicate and the experiments were repeated three times in time.

Leaf disc preparation

The first or second fully expanded leaves were removed from stock strawberry plants and transported directly to the laboratory so that the leaves were available for the bioassay in less than 4 hours. Immediately after collection, the leaves were placed in a tray lined with moist paper towels and the tray was closed to retain near 100% relative humidity (RH) and maintained at ~12°C. Only leaves with no visible signs of injury or symptoms of disease were collected. Whole leaves were disinfested by placement in a beaker with 2.5% Clorox for 3 min., leaves were rinsed 2-3 minutes in sterile DDI water three times, and leaves with any signs of bleaching were discarded. Strawberry leaf discs were then cut using a 15-mm stainless steal cork borer and placed in moisture plate until they were placed in the 24-well plate.

Pathogen and Inoculum Preparation

Three *Colletotrichum* isolates were evaluated for their ability to produce disease lesions under in vitro conditions. *Colletotrichum fragariae* (CF75), *C. fragariae* (CF63), and *C. gloeosporioides* (CG162) isolated from strawberry were evaluated for their ability to produce anthracnose symptoms on excised strawberry leaf discs. Each *Colletotrichum* isolates was grown on 1/2 strength potato dextrose agar (PDA) in 9-cm Petri dishes and incubated under (55±5 $\mu mols \cdot m^{-2} \cdot sec^{-1}$) Ushio Ultra 8 fluorescent lights (UFL-F17T8/741, Interlectric Corp., Warren, PA, USA) at 25º C as previously described (Abril et al., 2008). Conidia were harvest from 7- to 10-day-old cultures, and the aqueous conidial suspensions were filtered through sterile

Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA, USA) to remove hyphae. Conidial concentrations were determined photometrically from a standard curve based on absorbance at 625 nm (Espinel- Ingrof and Kerkering, 1991; Wedge and Kuhajek, 1998), and then stock suspensions were adjusted with sterile water to a concentration of 10^6 conidia / ml.

Surfactant and Inoculum Volume

Three concentrations of Tween 20 were evaluated for their effects on promoting pathogen infection of the leaf disk. Tween 20 concentrations of 0.3 %, 0.5 %, and 0.7% were prepared in 100 ml sterile distilled water. Conidial suspensions for inoculation were adjusted to a final concentration of 1.0×10^5 conidia / ml. Two inoculum volumes were also evaluated where the inoculum volume applied to each leaf disk was either 10 or 20 µl of either of the three fungal spore suspensions. After inoculation the 24-well were plates were sealed with paper-based tape and placed in the incubator under (55±5 $\mu mols \cdot m^{-2} \cdot sec^{-1}$) Ushio Ultra 8 fluorescent lights (UFL-F17T8/741, Interlectric Corp., Warren, PA) with a 12 h photoperiod in a growth chamber at 25º C for 6 days.

Fungicidal and phytotoxicity

Experimental compounds were evaluated in a dose-response format. Azoxystrobin that have protective and some curative activity were used as standards for comparison. A 20% ethanol:water solvent control is used in each study to solubilize lipophilic extracts. *Angelica sinensis* roots were purchased as a plant material from Jincheng Lingyi Drugstore, Weinan City, Shaanxi Province, China. *Origanim onites* essential oil was purchased from ALTES company, Turkey. Stock solutions containing 625, 1250, and 2500 ppm were prepared for azoxystrobin,

Origanum onites essential oil, *Angelica sinensis* essential oil and 20% ethanol solvent control in 50 ml capped test tubes.

Strawberry leaf disks prepared as previously described were dipped in antifungal test solutions for 3-5 seconds, excess solution was allowed to drain onto the edge of the test tube and then placed into the appropriate row and column of the 24-well plates. Plates were incubated over night at room temperature and inoculated the next morning with either 20 ul of conidial solution obtained from either *C. fragariae* (CF75), *C. fragariae* (CF63), or *C. gloeosporioides* (CG162). Percent healthy tissue, percent leaf necrosis, and percent phytotoxicity were determined at 5 days after inoculation by a Lemnatec Lemna HTS and SAW Scanalyzer analyzing software (LemnaTec GmbH, Wurselen, Germany). The instrument was set up to photograph (Sony DFW-SX900 camera) a 24-well plate containing the leaf disks using both top lighting and bottom lighting (8 W fluorescent tubes) and optimized for disk clarity and color. The software configurations were set to measure the area of each disk separately and classify the colors detected within each leaf and color classification was set to differentiate healthy tissue, bleached tissue, and necrotic lesions on the leaf surface.

Experimental Design

SAS (Statistical Analysis System, Cary, N.C.) was used to conduct analysis of variance and mean separation tests on all three experiments. A completely randomized design was used for all experiments. All factors were considered to be fixed effects, except for surfactant concentration in the surfactant experiment. Fisher's protected LSD was used to separate means (Steel and Torrie, 1980).

Results and discussion

24-well Leaf Disk Assay/ Salt solutions

Leaf color index obtained from visual evaluation of the leaf disks at 7 days indicated that ½ strength Hoaglands No. 2 basal salt media provided excised leaf discs with the best physiological support with a color index of 2.67. Murashige and Skoog basal salts demonstrated the lowest color index of 1.83. While MES salts and water had a color index of 2.42 and 2.83 respectively. However, MES and water did not gel sufficiently to provide adequate physical support to keep leaf disks (Figure 1) afloat for subsequent inoculation with the conidial solutions (Table 1).

Typical working concentrations for Phytagel™ are 1.5-2.5 g/L in plant tissue culture media and up to 10 g/L in microbiological media. Phytagel™ requires the presence of cations (especially divalent) for gelling to occur. Concentrations of calcium and magnesium contained in most plant tissue culture media are typically sufficient for gelation. Low-salt media formulations, especially those used in microbiological applications may require supplementation with additional calcium or magnesium salts (e.g., $CaCl_2$ or $MgSO_4$) or higher concentrations of Phytagel. Therefore we chose ½ strength Hoaglands No. 2 basal salt media containing 1% Phytogel as the best medium to conduct all subsequent 24-well leaf disk studies.

Pathogen type, surfactant, and inoculum volume

Colletotrichum fragariae (CF75), *C. fragariae* (CF63), and *C. gloeosporioides* (CG162) were each evaluated for their ability to produce disease lesions under *in vitro* conditions at three levels of Tween 20 (0.3 %, 0.5 %, and 0.7%). Mean necrosis indicated that *Colletotrichum fragariae* (CF75) and *C. gloeosporioides* (CG162) produced the highest number of lesions under

in vitro conditions and *C. fragariae* (CF63) produced the lowest number of lesions (Table 2). The best treatment combination was *C. fragariae* (CF75) where inoculum amended with 0.5% and 0.7% Tween 20 produced 16.72 and 15.53 % necrosis of the leaf disks respectively. *C. gloeosporioides* (CG162) showed no significant differences in leaf lesions between the three Tween 20 concentrations. Lesion development (necrosis) indicated that there was no difference between 10 or 20 µl inoculum volumes for either of the three *Colletotrichum* spore suspensions when applied to the leaf disk surface.

Fungicidal and phytotoxicity

Phytotoxicity evaluations were made visually at 12 hrs after treatment of the fungicidal solution or solvent control and prior to inoculation (Figure 1). *Origanum onites* essential oil above 1250 ppm demonstrated a 4+ phytotoxicity ranking and *A. sinensis* essential oil demonstrated 1+ phytoxicity ranking at 2500 ppm. Percent healthy tissue, % bleached tissue, and % necrosis of the leaf disk are presented in Table 3. The commercial fungicide azoxystrobin had the greatest level of disease control efficacy at 99% healthy tissue at 625 ppm and the lowest level of phytotoxicity (0.45%) at 2500ppm. Both *O. onites* and *A. sinensis* essential oils demonstrated phytotoxicity in a dose-dependent manner that was shown visually in Figure 1 and Figure 2, Plate 1a. Color analysis presented in Figure 2, Plate 1b show healthy area in green, diseased area in black and phytotoxicity in gray. *Origanum onites* essential oil was more phytotoxic to strawberry leaves than was *A. sinensis* essential oil. *Angelica sinensis* essential oil appeared to be a more effective antifungal solution and was effective against all three *Colletotrichum* species and had a lower % necrosis that did *O. onites* essential oil.

Conclusions

Discovery and evaluation of natural product fungicides is largely dependent upon the availability of miniaturized antifungal bioassays. Essentials for natural product bioassays include sensitivity to microgram quantities, selectivity to determine optimum target pathogens, and adaptability to complex mixtures. These assays should be relevant to potential pathogen target sites in the natural infection process of the host and applicable to the agrochemical industry. Bioassays need to take advantage of high-throughput technology to evaluate dose-response relationships, commercial fungicides standards, modes of action, and structure activity studies [18, 22].

The 24-well leaf disk bioassay has numerous advantages over conventional pot or field studies. The most important aspect of an *in vitro* assay is that testing is conducted under controlled conditions and miniaturized so that micro amounts of experimental compounds can be studied. The addition of digital photography and color imaging and analysis software make this assay a very powerful technique to evaluate lipophilic and other lead compounds directly on the leaf surface. Images can be stored electronically and analyzed at one's convenience rather that trying to conduct visual assessments in the field in the blazing sun or on a cold-wet windy day. Moreover, the opportunity to go back and adjust the color images and the analysis parameters to fine tune the data analysis that was frozen in time can not be replicated in the greenhouse or in the field. Identification of new potential lead compounds can be repeated quickly in time and real on-the-leaf-surface activity can be evaluated in high throughput formats and published in a reasonable time. There will still be a place for the studies evaluating multiple geographical locations and 3-year repeated studies but cost and efficiency of discovery research will continue to drive the need for the development of new techniques such as the 24-well leaf disk assay.

Acknowledgements

Our thanks to J'Lynn Howell for photographic expertise, Linda Roberston for technical assistance in performing various bioassays and Ramona Pace for her data management assistance. *Colletotrichum* isolates were obtained from Barbara Smith, USDA-ARS Southern Horticultural Laboratory Small Fruit Research Unit, Poplarville, MS.

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Table 1. Half strength Hoagland's No. 2, 2-N-morpholino ethanesulfonic acid (MES), Murashige and Skoog basal salt mixture (MS, and water (control) were evaluated for their ability to sustain excised leaf discs for 7-10 days. Mean leaf color index on a scale from 1-3 (1 = light green, 2 = medium green, 3 = dark green) was used to describe leaf chlorophyll content.

Salt Solution	Leaf Color Value (1-3)	Standard Error
H_2O	2.83 a	0.11
½ Hoagland's	2.67 a	0.22
M&S	1.83 ab	0.30
MES	2.42 b	0.19

Means followed by the same letter are not significant at $p<0.05$.

Table 2. Percent mean necrosis was used evaluate the ability of *Colletotrichum fragariae* (CF75), *C. fragariae* (CF63), and *C. gloeosporioides* (CG162) and three concentrations of Tween 20 (0.3 %, 0.5 %, and 0.7%) in the inoculum solution to produce disease lesions on 15 mm leaf disks.

Fungal isolate & % Tween 20		% Mean Necrosis	Standard Error
Cf63	0.3	7.34 cde	2.53
Cf63	0.5	4.84 efg	1.12
Cf63	0.7	5.78 def	2.05
Cf75	0.3	10.78 bcd	2.13
Cf75	0.5	16.72 a	0.93
Cf75	0.7	15.53 ab	8.52
Cg162	0.3	12.47 abc	3.27
Cg162	0.5	11.58 abc	2.02
Cg162	0.7	12.00 abc	3.20
H_2O	0.3	0.56 fg	0.00
H_2O	0.5	0.14 g	0.00
H_2O	0.7	0.00 g	0.00

Means followed by the same letter are not significant at $p<0.05$.

Plate 1

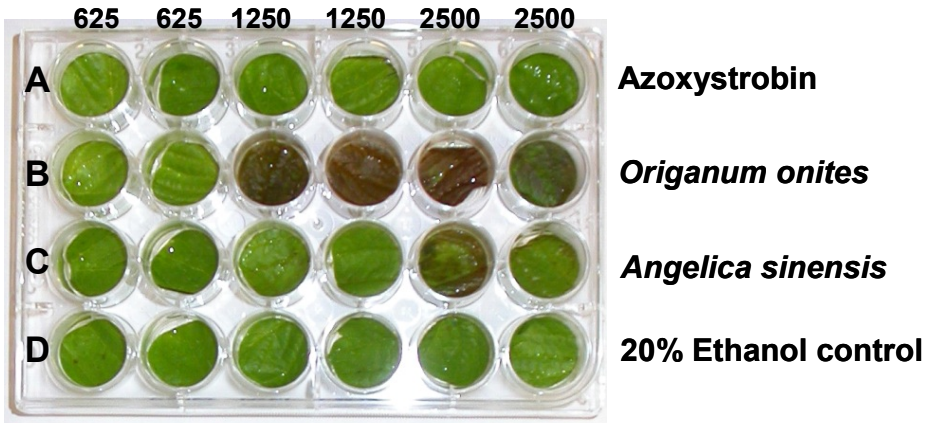

Plate 2

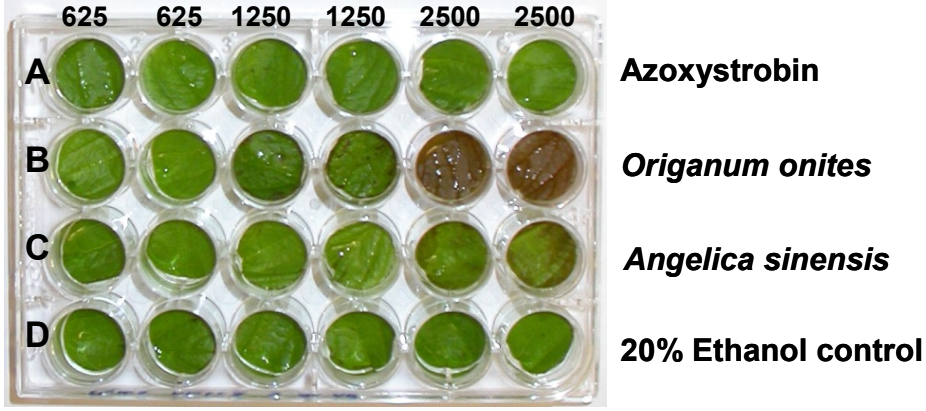

Figure 1. 15-mm excised leaf disks from anthracnose susceptible strawberry cultivar 'Chandler' demonstrate easy to visualize 4+ phytotoxicity of *Organium onites* essential oil above 1250 ppm. *Angelica sinensis* essential oil demonstrated 1+ phytoxicity at 2500 ppm. Row A contained azoxystrobin, row B contained *Organium onites* essential oil, row C contained *Angelica sinensis* essential oil at three concentrations. Row D contained 20% ethanol/water solvent control. Column 1 and 2 contain 625 ppm, column 3 and 4 contain 1250 ppm, and row 5 and 6 contain 2500 ppm of the respective test compounds or extracts.

Plate 1a

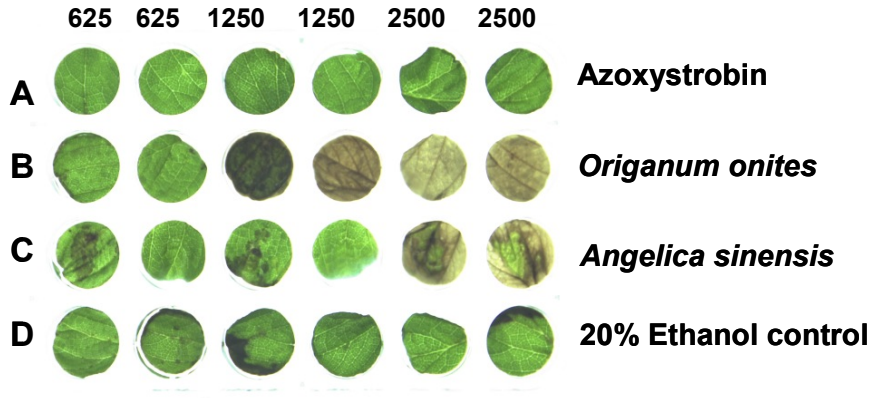

Plate 1b

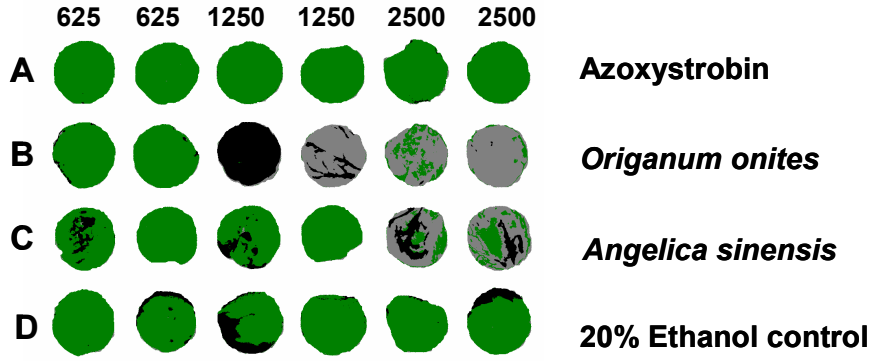

Figure 2. Plate 1a is a photographic image of 15-mm excised leaf disks from anthracnose susceptible strawberry cultivar 'Chandler' following 3-5 second dip in fungicide test solutions in a dose-response format and inoculated 12 hrs later with Colletotrichum fragariae isolate #75. Plate 1b is an image analysis of Plate 1a using the LemnaTec and SAW Scanalyzer analyzing software showing healthy tissue in green, diseased (necrotic) lesions in black, and bleached tissues (phytotoxicity) in gray.

Table 3. Percent healthy tissue, % leaf necrosis, and % phytotoxicity as determined at 5 days after inoculation by a Lemnatec Lemna HTS and SAW Scanalyzer analyzing software. *Colletotrichum fragariae* (CF75), *C. fragariae* (CF63), and *C. gloeosporioides* (CG162) were the test isolates used in these experiments.

	Azoxystrobin			Origanium onites			Angelica sinensis			
	625 ppm	1250 ppm	2500 ppm	625 ppm	1250 ppm	2500 ppm	625 ppm	1250 ppm	2500 ppm	Control
% Healthy Tissue										
Cf75	99.29	98.93	99.16	65.03	9.79	16.43	92.59	79.65	36.73	86.18
Cf63	99.55	99.51	99.64	96.17	7.32	20.94	96.02	72.20	16.18	92.22
Cg162	99.65	99.21	99.80	98.66	33.72	17.42	98.53	97.33	27.64	94.27
% Leaf Necrosis										
Cf75	0.40	0.55	0.39	25.81	29.78	1.02	6.49	8.78	9.59	13.01
Cf63	0.13	0.23	0.03	3.39	17.17	0.33	2.89	7.58	0.09	6.85
Cg162	0.04	0.40	0.09	1.13	15.97	7.19	1.05	1.42	0.91	4.85
% Phytotoxicity										
Cf75	0.31	0.52	0.45	9.16	60.44	82.55	0.92	11.57	53.67	0.80
Cf63	0.31	0.26	0.33	0.44	75.51	78.72	1.09	20.21	83.73	0.93
Cg162	0.31	0.39	0.11	0.21	50.31	75.39	0.42	1.25	71.44	0.88

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WORKING/SUBMITTED PAPERS

1. **Xiaoning Wang**, Mohamed M. Radwan, Amer H. Tarawneh, David E. Wedge, Stephen J. Cutler. Isolation and structure elucidation of secondary metabolites with antifungal activity from *Milogrante*. **2011**. In preparation.
2. Amer H. Tarawneh, Mohamed M. Radwan, **Xiaoning Wang**, David E. Wedge, Stephen J. Cutler. Two fatty acids with in vitro binding affinity for cannabinoid receptors from fungus UK-149. *Medicinal Chemistry Research*, **2011**. In preparation.
3. **Xiaoning Wang**, Mohamed M. Radwan, Jiangtao Gao, Amer H. Tarawneh, David E. Wedge, Horace G. Cutler, Stephen J. Cutler. Bioactive metabolites from *Cladosporium cladosporioides* against plant pathogens. *Journal of Agricultural and Food Chemistry*, **2011**. In preparation.
4. Jiangtao Gao, Mohamed Radwan, Francisco León, **Xiaoning Wang**, Olivia Dale, Susan P. Manly, Gui Ma, Afif Husni, Corey Gamelli, Frank Dugan, Robert Hill, Horace G. Cutler, and Stephen J. Cutler. Neocosmosins A-C: resorcyclic acid lactone derivatives from *Fusarium*

soloni with binding affinity for cannabinoid and opioid receptors. *Journal of Natural Products*, **2011**. Submitted.

PEER-REVIEWED JOURNAL ARTICLES

1. Jiangtao Gao, Mohamed M. Radwan, Francisco León, **Xiaoning Wang**, Melissa R. Jacob, Babu L. Tekwani, Shabana I. Khan, Shari Lupien, Robert A. Hill, Frank M. Dugan, Horace G. Cutler, Stephen J. Cutler. Antimicrobial and antiprotozoal activities of secondary metabolites from the fungus *Eurotium repens*. *Medicinal Chemistry Research*, **2011**. Accepted on September 12th.

2. **Xiaoning Wang**, Eman Habib, Francisco Leon, Mohamed Radwan, Nurhayat Tabanca, Jiangtao Gao, David E. Wedge, Stephen J. Cutler. Antifungal metabolites from the roots of *Diospyros virginiana* by overpressure layer chromatography. *Chemistry and Biodiversity*, **2011**, 8 (12): 2331-2340.

3. Jiangtao Gao, Francisco León, Mohamed M. Radwan, Olivia R. Dale, Corey A. Gemelli, Susan P. Manley, Shari Lupien, **Xiaoning Wang**, Robert A. Hill, Frank M. Dugan, Horace G. Cutler, Stephen J. Cutler. Benzyl derivatives with in vitro binding affinity for human opioid receptors and cannabinoid receptors from the fungus *Eurotium repens*. *Journal of Natural Products*, **2011**, 74 (7): 1636-1639.

4. **Xiaoning Wang**, David E. Wedge, Nurhayat Tabanca, Robert D. Johnson, Stephen J. Cutler, Patrick F. Pace, Barbara J. Smith, Ligang Zhou. Development of a miniaturized 24-well strawberry leaf disk bioassay for evaluating natural fungicides. *Natural Product Communication*, **2008**, 3(0): 1-6.

5. Nurhayat Tabanca, David E. Wedge, **Xiaoning Wang**, Betul Demirci, Kemal Husnu Can

Baser, Ligang Zhou, Stephen J. Cutler. Chemical composition and antifungal activity of *Angelica sinensis* essential oil against three *Colletotrichum* species. *Natural Product Communications*, **2008**, 3 (0): 1-6.

6. Youwen Gong, Xiujuan Zhang, **Xiaoning Wang**, Ligang Zhou. Effect of Essential Oil from *Zanthoxylum bungeanum* and *Phellodendron chinense* on Hyphal Morphology and Cell Wall Degrading Enzymes of Rhizoctonia solan. *Natural Product Research and Development* (in Chinese), **2008**, 20: 193-197.

CONFERENCE PRESENTATIONS AND POSTERS

1. **Xiaoning Wang**, Stephen J. Cutler. 2012. New leads to potential agrochemical agents from *Cladosporium cladosporioids*. **Poster**, 11th Annual Oxford International Conference on the Science of Botanicals (ICSB), Oxford, MS.
2. **Xiaoning Wang**, Stephen J. Cutler. 2011. Bioactive metabolites from *Cladosporium cladosporioides* against plant pathogens. **Platform**, MALTO Meeting, Houston, TX.
3. **Xiaoning Wang**, Stephen J. Cutler. 2010. Bioactivity directed isolation and characterization of antifungal compounds from *Diospyros virginiana*. **Poster**, American Society of Pharmacognosy & the Phytochemical Society of North America Meeting, Saint Petersburg, FL.
4. **Xiaoning Wang**, Stephen J. Cutler. 2010. Isolation and purification of antifungal metabolites from the roots of *Diospyros virginiana* by overpressure layer chromatography. **Poster**, MALTO Meeting, Oxford, MS.

5. **Xiaoning Wang**, David E. Wedge, Stephen J. Cutler. 2010. Isolation and purification of antifungal metabolites from the roots of *Diospyros virginiana* by overpressure layer chromatography. **Poster**, NCNPR Internal Poster Session, Oxford, MS.

6. **Xiaoning Wang**, David E. Wedge, Stephen J. Cutler. 2009. Development of a miniaturized 24-well strawberry leaf disk bioassay for evaluating natural fungicides. **Poster**, American Society for Horticultural Science Annual Conference, Saint Luis, MI.

7. **Xiaoning Wang**, David E. Wedge, Stephen J. Cutler. 2009. Development of a miniaturized 24-well strawberry leaf disk bioassay for evaluating natural fungicides. **Poster**, 7th Annual Oxford International Conference on the Science of Botanicals (ICSB), Oxford, MS.

8. **Xiaoning Wang**, David E. Wedge, Stephen J. Cutler. 2007. Development of a miniaturized 24-well strawberry leaf disk bioassay for evaluating natural fungicides. **Poster**, NCNPR Internal Poster Session, Oxford, MS.